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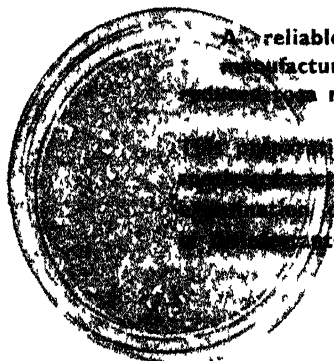
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## Occurrence of the M Substance of Type 28 Group A in Streptococci of Lancefield Groups B, C and G

By W. R. MAXTED

*Streptococcal Reference Laboratory, Central Public Health Laboratory, Colindale, London*

**SUMMARY:** Strains of streptococci belonging to Lancefield groups B, C and G were found to possess an antigen which is identical with the group A type 28 M antigen in its ability to absorb group A type 28 antibodies, and to stimulate the production of type 28 antibodies. The antigen also resembles the group A type 28 antigen in its sensitivity to pepsin and trypsin, being inactivated by pepsin but not by trypsin. The antigen does not appear to be the major type-specific antigen in some of the strains not belonging to group A.

Lancefield (1940) showed that the serological types of group A streptococci are each characterized by the possession of a type-specific protein antigen, the M substance. Occasionally,  $\beta$ -haemolytic streptococci are encountered which, although belonging to groups other than A, react with absorbed type-specific sera prepared against group A organisms. The nature of the antigen responsible for this cross-relationship has not so far been determined. It is the purpose of this paper to describe four such strains, one of which belonged to group B, two to group C and one to group G; all four strains had the M antigen characteristic of type 28 group A.

### *Material and Methods*

**Source of strains.** Strain 'Small' was Griffith's original group A type 28; strain 3962 group B came from a patient with a mild sore throat; strain D10 group C was also isolated from a sore throat. Strains 6074 group C and J23 group G came from the vaginae of patients with puerperal fever.

**Cultures.** For extraction or enzyme treatment the cells after 20 hr. incubation at 37° in 50 ml. of broth (Todd & Hewitt, 1932), were collected by centrifugation.

**Preparation of extracts.** Crude HCl extracts were prepared as described by Lancefield (1940). These extracts contained the group-specific C substance and the type-specific M antigen.

**Precipitation reactions.** The capillary technique of Swift, Wilson & Lancefield (1948) was used throughout.

**Preparation and absorption of antisera.** Antisera were prepared by inoculating rabbits with formalin-killed streptococci using a slight modification of Lancefield's technique. The strains were passaged a number of times through mice, and after confirming the presence of type 28 M substance, 250 ml. amounts of Todd-Hewitt broth were inoculated and incubated for 24 hr. at 37°. Formalin was then added to a concentration of 0.2% (v/v), and the flasks were left for 48 hr. at room temperature. The cultures were then centrifuged, the deposit

washed once in saline and concentrated by suspending in 17 ml. of 0.2% formol saline. Rabbits were given 0.5 ml. intravenously as a primary dose, rested 1 week, and then given 1.0 ml. on each of 8 successive days, and again rested for 1 week. After 8–10 weeks good precipitating sera were obtained. Three rabbits receiving the group G strain died before large bleedings could be taken; the smaller samples, however, gave good precipitation after absorption with a heterologous group G strain. All sera were absorbed with a heterologous strain of the same group to remove non-specific antibodies, the absorbing dose being 1 part of packed centrifuged deposit of cocci to 4 parts undiluted serum.

*The absorption of the type-specific antibodies from the group A type 28 serum by related strains of other groups*

The type 28 serum made with strain 'Small' was absorbed with type 8 cocci to remove non-specific antibodies. Strains 'Small' and 3962, D10 and J23 were then each used to absorb separate samples of the serum. Four control samples were absorbed each with a strain of either group A, B, C or G but unrelated to type 28; the samples were then tested for precipitins with extracts of strain 'Small' and of the other group strains related to it (Table 1). All the strains precipitating with type 28 serum removed the group A type 28 antibodies. The control samples retained their activity.

Table 1. *Precipitin reactions of a group A type 28 antiserum absorbed by strains of cocci containing the type 28 antigen, but belonging to various groups*

| M extracts prepared from strain | Antiserum absorbed by strain |                   |              |             |             |
|---------------------------------|------------------------------|-------------------|--------------|-------------|-------------|
|                                 | Group A type 8               | 'Group A' 'Small' | Group B 3962 | Group C D10 | Group G J23 |
| 'Small', group A                | + +/+ + *                    | —                 | —            | —           | —           |
| D10, group C                    | + +/+ +                      | —                 | —            | —           | —           |
| 3962, group B                   | + /+                         | —                 | —            | —           | —           |
| J23, group G                    | + /+                         | —                 | —            | —           | —           |
| 6074, group C                   | + +/+ +                      | —                 | —            | —           | —           |

\* The readings before the stroke were made after 2 hr. at 37°; those following the stroke after a further 24 hr. at 4°. Absorption with one strain of each group unrelated to type 28 failed to remove the precipitating antibody.

+ = a light uniform precipitate up the whole fluid column in the capillary tube.

+ + = a definite flocculation.

+ + + = heavy precipitate at the base of the column.

— = no precipitate.

*Effect of trypsin and pepsin on M extracts*

Lancefield (1948) has shown that the type-specific M substance of type 28 differs from the M substance of all the other types, except type 44, in that it resists the action of trypsin, though it is inactivated by pepsin. All other known M antigens in group A except type 44 are inactivated by trypsin as well as by pepsin. Therefore in order to identify further the type 28 antigen of these

group B, C and G strains, the action of pepsin and trypsin on their serological reactivity was investigated.

Duplicate samples of the centrifuged bacterial deposit were resuspended in 15 ml. amounts of either acetate buffer pH 2.5 or phosphate buffer pH 8.4. To the cells suspended in pH 2.5 buffer was added either pepsin in water to give a concentration of 1 %, or water; to the cells in pH buffer 8.4 was added

Table 2. *Precipitation reactions between type 28 M antibodies and M extracts, demonstrating inactivation by pepsin but not by trypsin*

| M extract prepared<br>from strain | Preliminary treatment of cells before preparation<br>of M extract |               |                             |               |
|-----------------------------------|---|---------------|-----------------------------|---------------|
|                                   | pH 8.4 buffer<br>and trypsin                                      | pH 8.4 buffer | pH 2.5 buffer<br>and pepsin | pH 2.5 buffer |
| 'Small', group A type 28          | +++/+*  | +++/++        | -/-                         | ++/++         |
| 3962, group B                     | ++/++   | ++/++         | -/-                         | ++/++         |
| D10, group C                      | ++/++   | ++/++         | -/tr                        | ++/++         |
| J23, group G                      | ++/++   | ++/++         | -/-                         | ++/++         |

\* Symbols as in Table 1.

either trypsin in water to give a concentration of 1 %, or water. The mixtures were incubated for 72 hr. at 37°, centrifuged and the deposits washed in saline. M extracts were made from each washed sample and tested for precipitation against group A type 28 serum. It will be seen (Table 2) that the serological reactivity of the M antigen from each strain was destroyed by pepsin and not by trypsin.

The results of the absorption and enzyme digestion tests suggested that it would be possible to prepare from these strains antisera which would precipitate with the M extract from group A type 28 strain 'Small'.

*Production of type 28 M antisera for strains 3962, D10 and J23*

The serum obtained after 8-10 weeks gave good precipitation with extracts of the immunizing strain. Samples of each serum were absorbed with a heterologous strain of the same group, to remove non-specific antibodies, then tested against extracts of (a) all the group A type strains, (b) other strains of the same group, and (c) all the strains showing relationship with type 28. All these sera reacted well with all the strains which had precipitated the group A type 28 serum (Table 3). No cross-reactions occurred with extracts of any of the other group A types, or with extracts of unrelated strains belonging to homologous or heterologous groups. The antisera so produced were after absorption indistinguishable from that produced by the group A type 28 strain 'Small'.

*The demonstration of another precipitating antibody in the serum made with group C strain D10*

It seemed possible that the sera contained another precipitating antibody type-specific within the particular group. An attempt was made to demonstrate this in the antiserum made to strain D10 group C. The group C non-specific

Table 8. *The specific antigenicity of type 28 M substances in strains of Str. pyogenes groups B, C and G*

| M extract prepared<br>from strain                              | Precipitin reactions with serum from rabbits<br>immunized with strain |                  |                  |                  |
|--|---|------------------|------------------|------------------|
|  | 'Small',<br>group A   | 3962,<br>group B | D 10,<br>group C | J 28,<br>group G |
| Group A types 1-30, and types 44<br>and 47                     | -/-*  | -/-              | -/-              | -/-              |
| 'Batty', group B; 'Azgazardah',<br>group C; 'Goodman', group G | -/-   | -/-              | -/-              | -/-              |
| 'Small', group A type 28                                       | +++++   | +++++            | +++++            | +++++            |
| 3962, group B  | +++   | +++              | +++              | +++              |
| D 10, group C  | +++++   | +++              | +++++            | +++++            |
| J 28, group G  | +++   | -/+              | +++++            | +++              |

\* Symbols as in Table 1.

antibodies were absorbed with strain 'Azgazardah' group C type 7. A further absorption with 'Small' group A type 28 removed all the type 28 antibodies. The serum was then tested for precipitins against HCl extracts of the strains from each group reacting with type 28 serum, and of four strains belonging to group C and not related to type 28 (Table 4). It will be seen that the serum D10 contained antibodies peculiar to the homologous strain. The strain 6074, which resembled strain D10 in other respects, did not possess this antigen. Only one of the early samples of the D10 serum gave this result.

Table 4. *To show the presence in strain D10, group C, type 28, of a specific precipitating antigen different from the type 28 antigen*

| M extracts prepared<br>from strain | Precipitin reactions with D 10 antiserum after<br>absorption with |                     |                          |                             |
|------------------------------------|---|---------------------|--------------------------|-----------------------------|
|                                    | Nil   | 'Small',<br>group A | 'Azgazardah',<br>group C | 'Small' and<br>'Azgazardah' |
| 'Small', group A                   | +++++*  | -/-                 | +++++                    | -/-                         |
| D 10, group C                      | +++++   | +/+                 | +++++                    | +++                         |
| 'Azgazardah', group C              | +/tr  | +/+                 | -/-                      | -/-                         |
| 6074, group C                      | +++++   | +/+                 | +++++                    | -/-                         |

\* Symbols as in Table 1.

Three other group C strains, K 64, 'Chestle' and 'Austin', failed to react with the serum after absorption with either 'Small' or 'Azgazardah'.

## DISCUSSION

Just as a T antigen of group A may be present in more than one type (Lancefield, 1940), so the M antigen of group A type 28 has now been shown to occur in strains from other groups, namely, B, C and G. Their behaviour in the laboratory and the clinical evidence supplied did not suggest that the possession of this antigen endowed these strains with greater pathogenicity than other streptococci of the same group. In spite of repeated passage through mice,

none of the strains became virulent, nor could they be used as infecting strains in passive protection tests in mice. The use of mucin to enhance infectivity for protection tests was ruled out, because it prevents contact of the cocci with the antibodies. It is probable that certain of the streptococci possessing the type 28 antigen have another major antigen more characteristic of the strain. It has long been known that serological types exist within group B (Lancefield, 1934, 1938; Stableforth, 1932), and that the type-specific antigen is a polysaccharide; the typing of such strains is a routine procedure in veterinary work. The strain 8962 was typed by this method as III*b*. Another group B strain D 186*a*, received from D. R. Lippmann, was originally typed as a group B type III, and this strain also possessed group A type 28 antigen. These two strains thus possessed three precipitating antigens, the group-specific and the type-specific polysaccharides, and the type 28 antigen, a protein. The strain of *Str. agalactiae*, B2, which was used to make the type III*b* antiserum with which strain 8962 reacted, did not show any relationship with type 28. It is possible also that the group C strain D10 possesses another antigen peculiar to it, which was not found in the other group C strains 'Chestle', 'Austin' and K64; nor in strain 6074, although this strain also contained the type 28 M antigen. The nature of this more specific antigen has not been investigated here, but Lancefield (1940-1) and Blakemore, Elliott & Hart-Mercer (1941) have found evidence of its protein nature.

Watson & Lancefield (1944) demonstrated that group A types 10 and 12 share a common M antigen, but it might be more correct to regard these two as one type, either 10 or 12, the T antigen of which varies for reasons not so far understood. It may be significant that the only M antigen so far found to be shared by strains in other groups should be the one which appears to have a different structure from the usual M antigen, as indicated by its resistance to tryptic digestion. The demonstration of the sharing of this unusual M antigen does not therefore contradict Lancefield's statements as to the specificity of the M antigen of group A. It will be interesting if type 44 M antigen is found in streptococci of other groups, since it also has the same characteristics as those of group A type 28 (Lancefield, 1943).

I wish to thank Dr V. D. Allison and Dr S. D. Elliott for advice and criticism, and also Dr R. C. Lancefield for supplying some of the strains.

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*lococcus aureus* to 15 % with the susceptible *Neisseria gonorrhoeae*. The majority of organisms appear to have a survival rate of about 50 %. Saline suspensions are usually less resistant than broth suspensions to freezing, the difference

Table 2. Viability tests on stored batches of dried cultures

Two tests were carried out. Test A: one tube from each batch was examined for viability. In the A columns are recorded the number of batches viable (+) or non-viable (—).

Test B: every tube in each batch which was negative in test A was examined for viability. The figures in the B columns record the number of batches in which every tube was non-viable.

| Organism and no. of strains examined | Period of storage (years) |    |   |     |    |   |     |   |   |     |    |   | Routine interval before redrying (years) |
|--------------------------------------|---------------------------|----|---|-----|----|---|-----|---|---|-----|----|---|--|
|                                      | 5-4                       |    |   | 4-3 |    |   | 3-2 |   |   | 2-1 |    |   |  |
|                                      | A                         |    | B | A   |    | B | A   |   | B | A   |    | B |  |
|                                      | +                         | -  |   | +   | -  |   | +   | - |   | +   | -  |   |  |
|                                      |                           |    |   |     |    |   |     |   |   |     |    |   |  |
| <i>Alcaligenes</i> spp. (5)          | 2                         | 0  | . | 5   | 0  | . | 2.  | 0 | . | .   | .  | . | 3  |
| <i>Vibrio comma</i> (3)              | 4                         | 2  | 2 | 2   | 2  | 0 | 6   | 2 | 2 | 5   | 2  | 0 | 1  |
| <i>V. foetus</i> (2)                 | .                         | .  | . | 5   | 0  | . | .   | . | . | 2   | 0  | . | 3  |
| <i>Pseudomonas</i> spp. (6)          | 1                         | 0  | . | 1   | 0  | . | 1   | 0 | . | 5   | 0  | . | 3  |
| <i>Staphylococcus</i> spp. (73)      | 2                         | 0  | . | 25  | 0  | . | 38  | 0 | . | 14  | 0  | . | 5  |
| <i>Neisseria</i> spp. (2)            | 1                         | 0  | . | .   | .  | . | 3   | 0 | . | .   | .  | . | 3  |
| <i>N. gonorrhoeae</i> (79)           | 43                        | 13 | 3 | 40  | 25 | 9 | 20  | 9 | 2 | 58  | 18 | 2 | 1  |
| <i>N. intracellularis</i> (36)       | 41                        | 3  | 0 | 27  | 11 | 2 | 24  | 7 | 0 | 30  | 8  | 1 | 2  |
| <i>Pasteurella</i> spp. (25)         | 4                         | 0  | . | 11  | 1  | 1 | 14  | 1 | 1 | 2   | 0  | . | 3  |
| <i>Brucella</i> spp. (20)            | 5                         | 0  | . | 12  | 1  | 0 | 8   | 0 | . | 1   | 0  | . | 5  |
| <i>Haemophilus</i> spp. (15)         | 3                         | 2  | 0 | 10  | 2  | 1 | 20  | 1 | 0 | 15  | 1  | 0 | 2  |
| <i>H. pertussis</i> (41)             | 9                         | 3  | 2 | 14  | 4  | 0 | 27  | 1 | 0 | 6   | 0  | 0 | 3  |
| <i>D. pneumoniae</i> (41)            | 10                        | 0  | . | 2   | 0  | . | 15  | 0 | . | 27  | 0  | . | 5  |
| <i>Streptococcus</i> spp. (128)      | 21                        | 0  | . | 41  | 0  | . | 54  | 0 | . | 39  | 0  | . | 5  |
| <i>Lactobacillus</i> spp. (18)       | .                         | .  | . | 10  | 2  | 0 | 1   | 0 | . | 8   | 0  | . | 3  |
| <i>Esch. coli</i> (113)              | 72                        | 6  | 0 | 17  | 1  | 0 | 51  | 1 | 0 | 103 | 0  | . | 3  |
| <i>Aerobacter</i> spp. (8)           | 2                         | 0  | . | 2   | 0  | . | .   | . | . | 6   | 0  | . | 3  |
| <i>Klebsiella</i> spp. (2)           | 2                         | 0  | . | 2   | 0  | . | 1   | 0 | . | 1   | 0  | . | 3  |
| <i>Serratia</i> spp. (4)             | 3                         | 0  | . | .   | .  | . | 4   | 0 | . | .   | .  | . | 3  |
| <i>Proteus</i> spp. (3)              | 2                         | 0  | . | 1   | 0  | . | 2   | 0 | . | .   | .  | . | 3  |
| <i>Salmonella</i> spp. (165)         | 42                        | 3  | 2 | 36  | 2  | 1 | 70  | 4 | 0 | 64  | 0  | . | 3  |
| <i>Shigella</i> spp. (65)            | 17                        | 6  | 2 | 22  | 1  | 0 | 26  | 3 | 2 | 15  | 0  | . | 2  |
| <i>Listeria</i> spp. (2)             | 1                         | 0  | . | .   | .  | . | .   | . | . | 1   | 0  | . | 3  |
| <i>Bacillus</i> spp. (20)            | 2                         | 0  | . | 8   | 0  | . | 3   | 0 | . | 10  | 0  | . | 5  |
| <i>Cl. novyi</i> (22)                | 1                         | 0  | . | 4   | 0  | . | 14  | 0 | . | 6   | 0  | . | 5  |
| <i>Cl. perfringens</i> (118)         | .                         | .  | . | 36  | 5  | 0 | 22  | 5 | 0 | 60  | 3  | 0 | 3  |
| <i>Cl. septicum</i> (40)             | .                         | .  | . | 27  | 0  | . | 17  | 0 | . | 19  | 0  | . | 5  |
| <i>Clostridium</i> spp. (32)         | 1                         | 0  | . | 14  | 0  | . | 7   | 0 | . | 21  | 0  | . | 5  |
| <i>C. diphtheriae</i> (61)           | 39                        | 2  | 0 | 21  | 1  | 0 | 54  | 0 | . | 2   | 0  | . | 3  |
| <i>C. pyogenes</i> (65)              | 48                        | 1  | 0 | 16  | 0  | . | 63  | 1 | . | 1   | 0  | . | 3  |
| <i>Corynebacterium</i> spp. (24)     | 3                         | 0  | . | 7   | 0  | . | 7   | 0 | . | 11  | 0  | . | 3  |
| <i>Mycobacterium</i> spp. (25)       | 3                         | 0  | . | 17  | 5  | 0 | 5   | 0 | . | .   | .  | . | 3  |
| <i>Actinomyces</i> spp. (5)          | 1                         | 0  | . | .   | .  | . | 3   | 0 | . | 1   | 0  | . | 3  |
| <i>E. rhusiopathiae</i> (5)          | 4                         | 0  | . | .   | .  | . | 6   | 1 | 0 | 1   | 0  | . | 3  |

being more marked with the organisms which are most susceptible. It is probable that the results for saline were weighted in favour of survival as the suspensions were not washed and therefore contained some soluble nutrients from the medium. The viability after freezing in other media, such as serum, 10 % gelatine, or M/60 phosphate buffer was also determined; some of these

media were as effective as broth, but none markedly superior. It appears that, provided protective substances are present, the initial loss on freezing is entirely related to the bacterial species. We have compared, with a number of organisms, the percentage survival after freezing to  $-17^{\circ}$  and to  $-78^{\circ}$  and have observed little significant difference in the numbers of organisms which survive.

Table 3. *Viability of organisms after freezing in CO<sub>2</sub> ice in broth and in saline*

0.2 ml. amounts of bacterial suspension frozen in small glass tubes; bottle method for viable counts.

| Organism                  | Percentage survivals after<br>freezing to $-78^{\circ}$ in |       |
|---------------------------|--|-------|
|                           | Saline   | Broth |
| <i>Staph. aureus</i>      | 100.0*   | 100.0 |
| <i>Esch. coli</i>         | 21.5   | 52.0  |
| <i>Sh. dysenteriae</i>    | 25.0   | 96.5  |
| <i>V. comma</i>           | 12.0   | 47.0  |
| <i>N. intracellularis</i> | 5.0  | 42.0  |
| <i>N. gonorrhoeae</i>     | 1.5  | 15.0  |

\* Presumably some disaggregation of clumps occurred.

*The survival of organisms after drying in broth and saline at room temperature or from the frozen state*

Table 4 summarizes the results of tests with six organisms of varying resistance to drying. *Staphylococcus aureus* represents the resistant class of Gram-positive cocci which remain viable in the dried state for many years. The survival rates may be as high as 100 %, and do not vary significantly whether the organism is dried in broth or saline, or at room temperature or from the frozen state. *Escherichia coli* and *Shigella dysenteriae* are intermediate in their resistance to drying, many cultures tending to die out after a few years as desiccates. The survival rate is still high, 20–60 %. On an average it is higher in cultures dried from the frozen state than in those dried at room temperature, although the difference is not marked. There is, however, a considerable diminution when saline is used as a suspending medium. *Neisseria intracellularis*, *N. gonorrhoeae* and *Vibrio comma* represent a class of organisms most sensitive to drying, the cultures beginning to die out after 2 years in the dried state. With these organisms the percentage survival is low, from 0.2 to 20 %, and drying the culture either at room temperature or suspended in saline kills either all or nearly all the cells.

*Effect of age of culture.* The age of the culture has some effect on its resistance to drying. *Shigella dysenteriae* was selected for test since it is intermediate in its resistance to drying. The organism was grown on agar and the total and viable counts made at intervals. The percentages of viable organisms at 5, 7, 9, 12, 24, 48 and 72 hr. were 11, 18, 11, 15, 6.8, 8.2 and 0.28 % respectively. The total counts were determined by opacity, using Brown's tubes and tables, and the viable counts by Miles & Misra's method (1938). In one experiment 7, 28 and 72 hr. cultures were dried, and in another 7, 28 hr. and 6-day cultures.

The first two periods were taken to represent approximately the beginning and end of the logarithmic growth stage and the last period the resting stage. Rather surprisingly the percentage survivals before and after drying were 20, 12.5 and 4.8 respectively in the first experiment and 24, 12.4 and 6.2 in the

Table 4. *Viability of organisms suspended in broth or saline and dried at room temperature or from the frozen state after freezing in CO<sub>2</sub> ice*

Viable counts by bottle method described in text.

| Organisms                 | Suspending medium | Temperature of drying | Survivals in different experiments (%) |
|---------------------------|-------------------|-----------------------|--|
| <i>Staph. aureus</i>      | Broth             | Room                  | 88.6, 51.5, 140.0, 60.0, 140.0         |
|                           |                   | -78°                  | 44.1, 42.4, 62.1, 127, 73.8            |
|                           | Saline            | Room                  | 34.9, 17.2                             |
|                           |                   | -78°                  | 44.1, 27.9                             |
| <i>Esch. coli</i>         | Broth             | Room                  | 43.8, 46.2                             |
|                           |                   | -78°                  | 64.6, 25.0, 42.4, 34.5, 31.9           |
|                           | Saline            | Room                  | 3.57, 1.2                              |
|                           |                   | -78°                  | 58.7, 46                               |
| <i>Sh. dysenteriae</i>    | Broth             | Room                  | 38.2, 29.1                             |
|                           |                   | -78°                  | 42.3, 10.4, 20.7, 28.2, 11.4           |
|                           | Saline            | Room                  | 0.74, 0.51                             |
|                           |                   | -78°                  | 2.3, 3.2                               |
| <i>N. intracellularis</i> | Broth             | Room                  | 0.69, 0.07, 0.15                       |
|                           |                   | -78°                  | 36.2, 19.7, 28.8                       |
|                           | Saline            | -78°                  | 0.003, 0.011, 0.03                     |
|                           |                   |                       |  |
| <i>V. comma</i>           | Broth             | Room                  | 5.5, 6.2                               |
|                           |                   | -78°                  | 4.7, 9.0, 2.6                          |
|                           | Saline            | -78°                  | 0.1, 0.003, 0.01                       |
|                           |                   |                       |  |
| <i>N. gonorrhoeae</i>     | Broth             | Room                  | Nil, Nil, Nil                          |
|                           |                   | -78°                  | 0.93, 1.33, 0.29                       |
|                           | Saline            | Room                  | Nil, Nil, Nil                          |
|                           |                   | -78°                  | Nil, Nil, Nil                          |

second. This suggests that young cultures are more resistant to drying, the sensitivity increasing somewhat with age. Similar results were obtained with *Escherichia coli*, but the differences were less marked.

### Storage loss

The initial loss on drying a bacterial culture is related chiefly to the species. In the present state of knowledge it is unlikely that it will vary much for a given organism dried by the various methods so far described, always provided a protective substance is present and the organism is dried from the frozen state. But in addition to the initial loss, there is a considerable and progressive storage loss which is the significant factor in maintaining a collection of bacterial desiccates. Table 5 gives the storage losses with *Esch. coli* and *Neisseria intracellularis* at different temperatures for a period of 6 months. With these particular desiccates, which did not receive a secondary drying, the storage loss was considerable and showed a fairly steep temperature coefficient.

At this stage an attempt was made to develop a method of determining the

probable storage loss without the long waiting period usually required. Accordingly counts were made on desiccates heated to 60, 80 and 100° for 1 hr. The decrease in viable count was considerable (Table 6). We obtained evidence that this loss runs parallel with the storage loss at lower temperatures. For example, the survival rates in a dried suspension of *N. intracellularis* after primary and secondary drying and storage for 1 month at room temperature

Table 5. *Effect of temperature on storage loss*

| Bottle method for viable counts. |                                    |                           |   |          |          |
|----------------------------------|------------------------------------|---------------------------|---|----------|----------|
| Organism                         | Surviving<br>drying<br>process (%) | Temperature<br>of storage | Percentage of surviving dried organisms<br>viable after |          |          |
|                                  |                                    |                           | 2 weeks   | 2 months | 6 months |
| <i>Esch. coli</i>                | 25.0                               | 4°                        | 83.1  | 53.1     | 28.5     |
|                                  |                                    | Room                      | 52.8  | 18.5     | 10.8     |
|                                  |                                    | 37°                       | 28.5  | 12.4     | 4.15     |
| <i>N. intracellularis</i>        | 17.7                               | 4°                        | 17.6  | 18.2     | 18.5     |
|                                  |                                    | Room                      | 7.1   | 7.1      | 4.6      |
|                                  |                                    | 37°                       | 3.0   | 0.002    | 0.006    |

were 0.5 and 3.2 %, and after 6 months less than 0.1 and 1.4 %. When these suspensions were heated immediately after completing the primary and secondary drying the survival rates after heating for 1 hr. at 60° were 0.13 and 10.0 % and after 1 hr. at 80° were less than 0.02 and 0.1 % with the primary and secondary dryings respectively. Similar results were obtained with a number of organisms, the differences between the two sets of figures varying considerably, according to the efficiency of the primary drying. It is likely that factors which lead to a high storage loss also result in a high loss on heating. This is a difficult point to prove decisively, and it is always possible that heating to high temperatures introduces factors which would not operate at room temperature. However, in practice it has proved a useful method of estimating probable storage loss.

The most obvious factor which might affect storage loss is the dryness of the resultant desiccate. With the routine method of drying and sealing it is probable

Table 6. *Effect of heat on viability of dried cultures after primary and secondary drying*

| Organism                  | Drying    | Percentage of dried organisms surviving<br>after 1 hr. at |        |          |
|---------------------------|-----------|---|--------|----------|
|                           |           | 60°   | 80°    | 100°     |
| <i>Esch. coli</i>         | Primary   | 0.18  | 0.001  | 0.0002   |
|                           | Secondary | 56.3  | 9.8    | 0.0008   |
| <i>Sh. dysenteriae</i>    | Primary   | 10.6  | 0.0081 | 0.000009 |
|                           | Secondary | 25.0  | 0.50   | 0.00001  |
| <i>N. intracellularis</i> | Primary   | 0.11  | Nil    | Nil      |
|                           | Secondary | 59.2  | 0.79   |          |
| <i>N. gonorrhoeae</i>     | Primary   | 4.4   | Nil    | Nil      |
|                           | Secondary | 48.3  | 0.14   | Nil      |

These are: damage to the cell by intracellular or extracellular ice formation, and protein denaturation due to changes in the physical state. We have observed that only two factors influence the survival rate after freezing: the species and the nature of the suspending medium. Since protective colloids, such as gelatine, which cannot pass through the cell wall, materially decrease the loss on freezing, it seems probable that extracellular factors, presumably ice formation, are responsible for death. We favour the hypothesis that the cell wall is punctured by extracellular ice crystals. Species differences would then be differences in the strength of the cell wall; in this connexion it may be more than a coincidence that organisms such as *Neisseria intracellularis*, *N. gonorrhoeae* and *Vibrio comma*, which are particularly susceptible to freezing, often show many degenerative forms on microscopical examination. The function of the protective colloid would then be to protect the organism against damage by ice crystals.

We have found two factors of paramount importance in avoiding a high storage loss. In the first place the cultures must be as dry as possible; secondly, they should be sealed in an atmosphere of dry nitrogen or *in vacuo*. It is possible that both the presence of oxygen and of traces of moisture are responsible for the higher storage loss when the tubes are taken out of the desiccator before sealing. Cultures that are not completely dry will not keep well in any case, but it is probable that even with completely dry cultures the storage loss would be less *in vacuo* or in nitrogen than in oxygen or air. The temperature of storage also has some effect, and dried cultures will survive longer in the cold room than at room temperature. One difficulty of sealing *in vacuo* is that when the tubes are opened the inrush of air is likely to contaminate the culture, or, when the tubes are sealed over a cotton-wool plug, to push the plug down to the bottom of the tube when it is opened; consequently we prefer sealing in dry nitrogen. The difficulty with nitrogen is that one cannot easily test for pinholes which may be present after the sealing process, as can be done when sealing *in vacuo*, by a high-tension vacuum tester. We have attempted to overcome this difficulty by including a few granules of dried silica gel over the cotton-wool plug. This acts as a secondary desiccant and in the presence of a leak absorbs moisture from the atmosphere, changing colour and acting as an indicator of the faulty sealed tubes. In practice the imperfectly sealed tube has been rare.

One of the difficulties in keeping a collection of desiccates is to know how any particular drying will survive, and a routine test to ascertain this would be of extreme value. We have devised such a test, which in our limited experience is promising. The test consists in heating one tube of the batch in a water-bath for 1 hr. at 80° and subcultivating. The number of organisms dried by the routine process is of the order of  $10^9$ . Should the drying be satisfactory, then after heating the number of organisms which survive should be of the order of  $10^7$  or more in the resistant and intermediate groups, and  $10^4$  in the susceptible group. If the drying is unsatisfactory the survivals are from 100 to more than 1000 times less (see Tables 6 and 7). Consequently if about 2 ml. of broth are added to the heated tube and 1 drop (about 0.05 ml.) is

placed on an agar medium plate, then with a successful drying there should be confluent growth with the resistant and intermediate groups and a fairly large number of colonies with the susceptible group. With an unsuccessful drying there would be isolated colonies with the first two groups and no growth with the last.

Our thanks are due to Mr E. Harris for technical assistance in maintaining the bacterial collection.

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## The Production of Penicillin in Surface Culture, using Chemically Defined Media

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**SUMMARY:** The production of penicillin by *Penicillium notatum* in surface culture on a number of chemically defined media is described. Good results were obtained with media containing lactose, glucose, acetic, citric and phenylacetic acids, ammonium sulphate and inorganic salts. The yield of penicillin was raised by further addition of starch and an aliphatic base such as ethylamine. In the latter instance the penicillin yield was about the same as that obtained with media containing corn-steep liquor.

A suitable addition of mineral salts should include magnesium, potassium, iron, zinc, copper, manganese, cobalt, phosphate and fluoride.

The addition of starch and ethylamine increased the growth of the mould and produced crinkled felts similar to those obtained with corn-steep liquor. These media have a composition which is in many ways comparable with that of media containing corn-steep liquor, and it seems likely that corn-steep liquor owes its effectiveness to the fortuitous presence in it of several types of substances rather than to one substance.

Although media containing lactose and corn-steep liquor (hereafter referred to as CSL) are usually used for the preparation of penicillin, considerable interest has been shown in chemically defined media. Apart from their possible technical value, such media may be a means of throwing light on the mechanism of penicillin formation.

A number of chemically defined media has already been described which are claimed to give high yields of penicillin in surface culture. White, Krampitz & Werkman (1945) described a medium containing lactose, glucose, arginine, histidine and glutamic acid; this recipe was based on analyses made on CSL. Cook & Brown (1946, 1947) subsequently described media containing gelatine or leucine and sugars, and also used media containing a variety of simple and complex nitrogen sources. More recently a patent specification (Glaxo Laboratories Ltd., British Drug Houses Ltd., Bide, Mead, Smith & Stack, 1947), has been published describing a number of more or less chemically defined media, which contained cystine or protein hydrolysates, in addition to phenylacetic acid or some similar substance with a suitably substituted benzyl group. A group of workers at Wisconsin has described media for the production of penicillin in submerged culture (cf. Jarvis & Johnson, 1947; Higuchi, Jarvis, Peterson & Johnson, 1946); these media contained, in addition to carbohydrates, lactate, acetate, ammonia and frequently phenylacetamide or a similar substance. The reason for the high productivity of media containing CSL has not yet been completely explained; current opinion is that it may be due to the presence of certain amino-acids in conjunction with substances like



$\beta$ -phenylethylamine, which CSL is known to contain (Glaxo Laboratories Ltd. *et al.* 1947), and which enter directly into the penicillin molecule, thus stimulating production.

When the present work was begun we thought that the amino-acids were not perhaps of specific importance, and that they could probably be replaced by a suitable organic acid or by a mixture of acids. The stimulation of growth of *Phycomyces blakesleeanus* by the addition of organic acids to the medium had already been described by several groups of workers (e.g. Leonian & Lilly, 1940), while the Wisconsin workers mentioned above had already described their use for submerged cultures of *Penicillium*. Marloth (1981) had also shown that the addition of citrate to media containing sucrose increased the growth of two species of *Penicillium*.

Preliminary experiments showed that media containing fairly high concentrations of citric acid gave fairly good yields of penicillin, and a basal medium was devised containing lactose, glucose, acetic acid, citric acid, ammonium salts, phenylacetic acid and metallic salts. This medium gave yields of 70–90 units of penicillin/ml., but these were lower than those obtained with media containing CSL, which usually give 100–140 units/ml. under our conditions of culture. The weight of mycelium produced was also less, and the felts were smooth and wavy rather than crinkly and without the cracked and pitted undersurface usually obtained with CSL. In order to enrich the medium without the use of protein hydrolysates or amino-acids, ethylamine or other aliphatic bases together with starch were added. This resulted in the production of thick, crinkly felts with pitted undersurface which are characteristic of CSL growths. The yield of penicillin was also raised to levels obtained with CSL. In the course of our experiments tests were made of the effects of varying the concentrations of the various ingredients, carbohydrates, organic acids, ammonium concentration, etc. Difficulties which occurred at one period also led to a brief investigation of the inorganic requirements of the mould.

## EXPERIMENTAL

*Cultural conditions and methods.* The organism used throughout this work was derived from the original Peoria strain *Penicillium notatum*, 1249 B21 (the culture used is labelled by us M2). For the inoculation of cultures the mould was grown on glycerol-molasses-peptone agar (cf. Frank, Calam & Gregory, 1948), and the spores brought into aqueous suspension by milling with glass beads. One ml. of suspension, containing  $5 \times 10^8$  spores, was added to each flask of medium under test. On occasion we have also used suspensions containing  $5 \times 10^7$  spores/ml.; these have given equally satisfactory results.

Media under examination were put up in 250 ml. Pyrex conical flasks containing 100 ml. of medium. The depth of medium is thus 25 mm., which is similar to that used in commercial practice. Three or four replicate flasks were normally employed. All flasks, etc., were sterilized by autoclaving for 20 min. at 15 lb./sq.in., and after inoculation were incubated, stationary, at 28–24°.

Penicillin was assayed by the cylinder-plate method normally used in these laboratories, using *B. subtilis* as test organism (cf. Foster & Woodruff, 1944). For assay, metabolism solutions were diluted twenty-fold with water. Differences of 5–10 % are distinguishable by this procedure.

*Media.* Preliminary experiments indicated that a medium of the following composition was likely to be of interest, and experiments were therefore based on it (all figures as % w/v): lactose, 8; glucose, 1; citric acid, 1; acetic acid, 0.25; phenylacetic acid, 0.05; ammonium sulphate, 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05;  $\text{KH}_2\text{PO}_4$ , 0.1;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.001;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.001;  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 0.002; NaF, 0.001; pH adjusted to 5.6–5.9 with potassium hydroxide; water to 100. This recipe will be referred to as Basal Medium. Throughout, the figures given represent the total concentration in the medium and are given as % w/v. As far as possible A.R. or B.P. materials were used for medium preparation. Shortages necessitated the use of glucose chips instead of A.R. glucose in many experiments.

## RESULTS

### *Inorganic components*

There is considerable evidence in the literature that the penicillin-producing *Penicillia* are fairly exacting in their mineral requirements. Knight & Frazier (1945) have shown that with their chemically defined medium, penicillin titres were increased when ash from CSL was added. Pratt (1945) showed that there were optimal concentrations for  $\text{MgSO}_4$ ,  $\text{KH}_2\text{PO}_4$  and  $\text{NaNO}_3$  when added to media containing CSL, and Cook, Tulloch, Brown & Brodie (1945) found that the ash from certain pea fractions was beneficial to penicillin production. More recently Koffler, Knight & Frazier (1947) made a further study, using *P. chrysogenum* X-1612, of the causes of the stimulatory effect of the ash of CSL mentioned above. They concluded that Fe and phosphate were the active constituents of the ash. The presence of more than 2 p.p.m. Cu prevented the formation of penicillin, but the addition of 1 p.p.m. Fe antagonized this effect and good yields of penicillin were obtained. Pratt & Dufrenoy (1947) have also shown that *P. chrysogenum* X-1612 gave improved yields of penicillin when traces of Cr and Al were added to chemically defined media.

In our earliest experiments additions of inorganic salts to the media consisted of small quantities of  $\text{KH}_2\text{PO}_4$  and the sulphates of Mg, Fe, Zn, Cu and Mn (referred to as GA salts). It appeared later that these were not adequate and that we were relying on casual impurities in the ingredients to supply the requirements of the organism.

In some of our cultures poor growth of the mould was accompanied by low titres; 30–50 units/ml. instead of 70–90 units/ml. Dried felts weighed 0.4–0.5 g./flask instead of 0.7–0.8 g./flask. This behaviour suggested that the mould was being deprived of necessary inorganic nutrients or trace elements, especially because there was usually an increase in the titre when the ash from CSL was added to the medium. Thus in one experiment titres were raised from 51 to

78 units/ml. on the 10th day by addition of the ash from the equivalent of 0.67 % (w/v) of CSL solids.

Spectrographic analyses of ash of a sample of British CSL showed that it contained considerable quantities of Mg, K, Na and P. There were small amounts of Fe, Mn and B and traces of Al, Cu, Zn, Si, Sn and Ga. On the other hand, Sc, Cb, Co, Ni and Mo were absent. Spore suspensions, as used for inoculation, contained Na, Mg, and lesser quantities of Si, Mn, Fe, and probably B; Sn, W, Mo, Cb, Sc, Co and Ni were absent.

These results for the analysis of CSL ash do not differ substantially from those described by Koffler *et al.* (1947), except that the American workers covered a somewhat wider range of elements. The American CSL contained traces of Co and Ni, whereas these were absent in the British materials. Quantitative analyses of four other batches of British CSL gave the following results: Mg, 0.12–0.38 %; K, 2–2.4 %; phosphate, 2.5–3.6 %; Cu, 4–40 p.p.m.; Zn, 44–65 p.p.m.; Fe, 250–325 p.p.m., based on the liquid which contained 85 % (w/v) solids.

Experiments were carried out in which the effect of adding various elements was tested. The elements tested included Cu, Zn, Fe, Mn, Mg, Mo, W, F, I, Co, Sn, Rb, Ag and Al. Of these all except the last four gave results of interest. The results of a typical experiment are given to illustrate the methods used. To Basal Medium, with no salt addition except  $\text{Na}_2\text{SO}_4$ ,  $\text{NH}_4\text{NO}_3$ , KOH and  $\text{KH}_2\text{PO}_4$ , there were added all combinations of 1 % (v/v) of solutions which contained the following salts in 1 l. of water:

- |  |   |
|--|---|
| (a) $\text{CuSO}_4$ , $\text{ZnSO}_4$ , $\text{MnSO}_4$ , 1 g.; $\text{FeSO}_4$ , 2 g. | (d) NaF, KI, 1 g.   |
| (b) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 g.                                  | (e) $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1 g. |
| (c) Na molybdate and Na tungstate, 1 g.  |   |

Groups of ingredients were used in this way to reduce the number of treatments, which was 32. The mean titre was 57 units/ml., the group with no additions having a titre of 48 units/ml. The mean effects of the five treatments were: (a) +2 units/ml.; (b) +6; (c) –3; (d) +5; (e) +4. There were some positive interactions, so that with suitable additions titres were of the order of 70–75 units/ml.

On the basis of this and similar experiments a salt addition was devised in which the following supplements (quoted above in the recipe for the Basal Medium) were made:  $\text{KH}_2\text{PO}_4$ , 0.1 %;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 %;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.001 % each;  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 0.002 %; and NaF, 0.001 %. The concentrations of the various elements were determined as a result of separate experiments. Iodide was found to be deleterious. When media were made up with this salt the ash of CSL ceased to have a stimulatory effect.

With most batches of ingredients, media containing the original GA salt addition gave results similar to those quoted for the Basal Medium, which contains the above salt additions. This is no doubt due to the accidental presence of the necessary elements in the various ingredients. Thus in a certain experiment the addition of 5 p.p.m. fluorine had no effect; analysis showed, however, that the unsupplemented medium already contained 8 p.p.m.

The experiments described above do not define exactly the inorganic requirements of the mould, but show that growth and penicillin production are satisfactory when Mg, K, P, Fe, Cu, Mn, Zn, Co and F are added to the medium. It is possible that the elements in the ash of CSL that produce the stimulatory effect are different from these, but that one of them is a satisfactory substitute. Our results differ from those of Koffler *et al.* (1947), especially in that low titres were obtained even in the presence of Fe. However, it is possible that the two strains of *Penicillium*, 1249 B21 and X-1612, differ in their mineral requirements.

### Organic components

*Citric and acetic acid.* The effect of varying the concentration of citric acid in the Basal Medium was first studied. The use of citric acid in chemically defined media had already been described by Clayton, Hems, Robinson, Andrews & Hunwick (1944), but in that instance low concentrations of the acid (0.2 % sodium citrate) were used. Higher concentrations than this are necessary if high yields of penicillin are to be obtained. Thus, in one experiment

Table 1. *Penicillin production with citric and acetic acid*

The Basal Medium was used with 0.5 %  $\text{NH}_4\text{NO}_3$  instead of  $(\text{NH}_4)_2\text{SO}_4$  and sugars as indicated.

| Mean titres (units/ml.) on 6th and 7th days |                 |       |      |     | Mean titres (units/ml.) on 8th and 9th days |                 |       |      |     |
|---|-----------------|-------|------|-----|---|-----------------|-------|------|-----|
| Citric acid (%)                             | Acetic acid (%) |       |      |     | Citric acid (%)                             | Acetic acid (%) |       |      |     |
|   | 0               | 0.125 | 0.25 | 0.5 |   | 0               | 0.125 | 0.25 | 0.5 |
| 1. With 2 % glucose and 2 % lactose.        |                 |       |      |     |   |                 |       |      |     |
| 0   | 0               | 0     | 25   | 25  | 0   | 0               | 19    | 35   |     |
| 0.5   | 28              | 44    | 60   | 76  | 0.5   | 33              | 48    | 62   | 71  |
| 1.0   | 56              | 72    | 64   | 51  | 1.0   | 51              | 68    | 76   | 76  |
| 1.5   | 59              | 61    | 70   | 44  | 1.5   | 74              | 74    | 79   | 73  |
| Mean of 6 best titres 67 units/ml.          |                 |       |      |     | Mean of 6 best titres 75 units/ml.          |                 |       |      |     |
| 2. With 1 % glucose and 3 % lactose.        |                 |       |      |     |   |                 |       |      |     |
| 0   | 0               | 0     | 21   | 21  | 0   | 0               | tr    | 40   | 29  |
| 0.5   | 17              | 23    | 38   | 53  | 0.5   | 24              | 37    | 49   | 61  |
| 1.0   | 37              | 34    | 61   | 61  | 1.0   | 42              | 50    | 78   | 70  |
| 1.5   | 45              | 50    | 58   | 57  | 1.5   | 65              | 74    | 78   | 84  |
| Mean of 6 best titres 58 units/ml.          |                 |       |      |     | Mean of 6 best titres 75 units/ml.          |                 |       |      |     |

the titres at 9 days were 23, 62, 83 and 91 units/ml. with 0.2, 0.5, 0.8 and 1.2 % respectively of citric acid. Table 1 illustrates another experiment, in which various concentrations of acetic and citric acid were employed. It will be noted that in the test of citric acid, titres rose slightly more rapidly with the 2 % glucose 2 % lactose mixture, but that otherwise the two sets gave similar results. The highest titres were obtained with 1.0–1.5 % citric acid.

No acid or mixture of acids has so far been found that is quite so effective as citric acid in chemically defined media, although some give quite good

results. The acids tested included glutamic, succinic, malic, lactic and tartaric acids, as well as mixtures of glutamic acid and either succinic acid or butyleneglycol.

Many substances can replace acetic acid. These include: succinic acid, oxalic acid, nucleic acid, acetylcholine, glutamic acid, diethylamine, sarcosine, glycerol, ethanol, *iso*-propanol, *n*-butanol, aspartic acid, hydroxypropionic acid, glutamic acid, oxalic acid. Benzylalcohol, glycine, glycolic acid, methanol, alanine, lactic acid and *iso*-caprylic alcohol were unsatisfactory.

**Carbohydrates.** The use of lactose follows current practice in penicillin work (Moyer & Coghill, 1946). The addition of glucose was found necessary by us if rapid growth were to be obtained. The concentrations used, lactose 3% and glucose 1%, were chosen empirically, and such tests as have been carried out suggest that these concentrations are as good as any, though in Table 1, where media are compared containing two different mixtures of the sugars, there is a suggestion that penicillin production is slightly more rapid with the 2:2 than with the 3:1 combination. Initially A.R. glucose was used, but this later became unobtainable and commercial glucose chips were used thereafter. Comparative experiments showed that both grades of glucose gave satisfactory results. Glycerol can replace glucose in synthetic media, but ethyleneglycol and 2:3-butyleneglycol are unsuitable for this purpose. The use of dextrans or starches as carbohydrates is discussed below. In another experiment the effect of varying the quantity of glucose in the presence and absence of acetic acid was tested. In the presence or absence of acetic acid an increase in the concentration of glucose from 0.5 to 1% raised the titre on the 8th day by about 40%. A further increase to 2% produced no improvement. Media containing starch and ethylamine give a different effect (see below, Table 5).

**Ammonia, nitrate, sulphate and the use of potassium and sodium for neutralization.** In a survey experiment all combinations of these factors were tested using three concentrations of each. The Basal Medium contained 2% lactose, 2% glucose, 1.5% citric acid, 0.125% acetic acid, 0.03% phenylacetic acid and salts. To these were added all combinations of:

|                                |      |      |     |
|--------------------------------|------|------|-----|
| Sulphuric acid (%)             | 0.5  | 1.0  | 2.0 |
| Nitric acid (%)                | 0    | 0.5  | 1.0 |
| Ammonia (as $\text{NH}_3$ , %) | 0.08 | 0.16 | 0.5 |

The media were adjusted to pH 5.7 with mixtures of sodium and potassium hydroxides in the ratios 1:0, 1:1, 0:1. The experiment was thus a  $3 \times 3 \times 3 \times 3$  factorial design with 81 combinations.

The average penicillin titres for the different concentrations of ammonia and for the different neutralization mixtures were:

$\text{NH}_3$  (%): 0.08, 0.16, 0.5; 38, 71, 81 units/ml.  
Na/K:: 1:0, 1:1, 0:1; 43, 49, 47 units/ml.

The ammonia concentration of 0.16% was thus optimal, and potassium or a mixture of sodium and potassium was better for neutralization than sodium alone. Taking the results for the media containing 0.16%  $\text{NH}_3$  neutralized with potassium only, the effect of using different concentrations of nitrate and

sulphate is shown in Table 2. Sulphuric acid was best at 0.5 %, and nitric acid was better at 0.5 % than at 0 or 1 % with this concentration of sulphuric acid.

The two basal media commonly used, incorporating 0.5 % of either ammonium sulphate or ammonium nitrate, contained 0.124 and 0.106 %  $\text{NH}_3$  respectively. As it was thought that the former gave slightly higher titres the matter was investigated in two experiments, over a range of 0.05–0.3 %  $\text{NH}_3$ . The best titres were obtained with about 0.13 %  $\text{NH}_3$ . Mycelial weights were highest with rather more ammonia (*c.* 0.22 %). The presence or absence of nitrate (0.5 %) did not affect these optima, nor did a change in the concentration of citric acid from 0.9 to 1.5 %. In one experiment titres were the same with or without nitrate, in the other nitrate increased the average from 70 to 83 units/ml.

Table 2. *Effect of concentration of nitrate and sulphate on penicillin production*

| Nitric<br>acid (%) | Sulphuric acid (%) |     |     |
|--------------------|--------------------|-----|-----|
|                    | 0.5                | 1.0 | 2.0 |
|                    | Titres (units/ml.) |     |     |
| 0                  | 73                 | 72  | 61  |
| 0.5                | 92                 | 78  | 59  |
| 1.0                | 70                 | 78  | 59  |

From these experiments it was concluded that the addition of about 0.5 % ammonium sulphate is suitable and that neutralization with potassium is advantageous. The concentration of ammonia is rather sharply optimal at 0.13 %. Nitrate usually increased the production of penicillin slightly, generally about 10 %.

*Phenylacetic acid and  $\beta$ -phenylethylamine.* The great increase in penicillin production which follows the addition of phenylacetic acid or other suitable compounds containing the benzyl radical is now well known (*cf.* Glaxo Laboratories Ltd. *et al.* 1947). When phenylethylamine is used it is probably oxidized before utilization. It was thought that this substance or a combination of it with phenylacetic acid might give better results than the acid alone, since its action might be more prolonged. An experiment showed that although the titres obtained with  $\beta$ -phenylethylamine were slightly higher than those with phenylacetic acid, the difference between the two was insignificant.

*Sulphur sources.* In our experiments sulphate has normally been used as a source of sulphur. Cystine and methionine are also satisfactory sources of sulphate, though in our hands they have not proved superior to sulphate. A detailed study of the sulphur metabolism of *P. notatum* has recently been made and will be published soon.

*Starches.* The idea of adding starches and organic bases arose from a consideration of CSL, which, it was thought, might well contain substances of these types. A considerable number of different types of starches and dextrans exist, and an experiment was carried out in which several different kinds were added to the Basal Medium (with 0.5 %  $\text{NH}_4\text{NO}_3$  and 1.5 % citric acid). Of

the starches 1 or 8 % were added with 8 % lactose and 1 % glucose respectively. The two sets were inoculated and incubated side by side. The results of this experiment are shown in Table 8.

Table 8. *Penicillin production by media containing dextrins instead of glucose or lactose*

| Carbohydrate                               | Days              |    |    | Felt<br>wt./flask<br>(g.) |
|--|-------------------|----|----|---------------------------|
|  | 8                 | 9  | 10 |                           |
|  | Titre (units/ml.) |    |    |                           |
| (a) With 8 % lactose and 1 % carbohydrate. |                   |    |    |                           |
| Soluble starch (B.D.H. Ltd.) A.R.          | 46                | 22 | 82 |                           |
| White maize dextrin                        | 35                | 57 | 79 |                           |
| Yellow maize dextrin                       | 15                | 27 | 35 |                           |
| British gum                                | 39                | 54 | 63 |                           |
| British gum (dark)                         | 24                | 41 | 58 |                           |
| Chlorinated gum 250                        | 51                | 84 | 77 |                           |
| Chlorinated gum 300                        | 56                | 63 | 67 |                           |
| Wet acid dextrinized gum                   | 55                | 71 | 77 |                           |
| Alkali treated gum                         | 37                | 64 | 68 |                           |
| Glucose (crude) chips                      | 68                | 82 | 88 | 0.92                      |
| Pure glucose                               | 68                | 84 | 88 |                           |
| (b) With 3 % carbohydrate and 1 % glucose. |                   |    |    |                           |
| Soluble starch (B.D.H. Ltd.) A.R.          | 84                | 89 | 92 | 0.98                      |
| White maize dextrin                        | 59                | 71 | 77 | 1.12                      |
| Yellow maize dextrin                       | 38                | 50 | 70 | —                         |
| British gum                                | 45                | 58 | 65 | —                         |
| British gum (dark)                         | 39                | 52 | 59 | —                         |
| Chlorinated gum 250                        | 28                | 63 | 71 | 1.08                      |
| Chlorinated gum 300                        | 29                | 55 | 78 | —                         |
| Wet acid dextrinized gum                   | 47                | 72 | 76 | —                         |
| Alkali treated gum                         | 61                | 72 | 92 | —                         |
| Lactose B.P.                               | 68                | 84 | 88 | 0.84                      |

It was apparent from this experiment that soluble starch and the alkali-treated gum could be substituted for lactose. The second set of media gave higher titres than the first. It was noticed that with most of the media containing 8 % polysaccharide the growth habit of the mould resembled that on CSL to such an extent that it was almost impossible to distinguish mycelial felts of similar age grown on the chemically defined medium containing the chlorinated gum 250 from those grown on a normal CSL medium. With many of the starches at the 8 % level the mycelial felt weights were also higher than with lactose. Taken together the results of these experiments show that, with suitably chosen mixtures, starch or dextrin can replace lactose in this type of chemically defined medium, but that the titres are not much enhanced by these additions.

*Organic bases.* A preliminary experiment with some organic bases indicated that the addition of ethylamine to media as a source of nitrogen might be useful. It also showed that ethanolamine, nitrate and urea were less satisfactory, while triethylamine, hexamethylenediamine, octadecylamine and dibutyl-

amine-propylamine failed to support growth. Ethylamine was therefore selected for further trial.

A large experiment was set up in which three mixtures of sugars and three mixtures of citric and acetic acid were tested with the addition of ammonia (0, 0.06 and 0.18 % as  $\text{NH}_3$ ), ethylamine (0, 0.3 and 0.6 %) and starch (0, 1 and 2 %). The Basal Medium containing 2 % glucose and 2 % lactose was the best of those tested, and addition of citric acid in the usual way seemed advisable. Ammonia enhanced the titres as starch did, 1 or 2 % giving about the same results. Ethylamine was best at 0.3 %.

On the basis of these and a few other experiments it was concluded that as a basis for investigation the Basal Medium given above, with the addition of 0.3 % ethylamine and 1.5 % starch, would be suitable. This medium contains too many components for a trial of every combination, so the effect on penicillin titre of varying the composition of the medium was tested in a number of small experiments dealing with only a few of these components. The factors investigated included the effect of varying the concentrations of ethylamine, starch, glucose, acetic acid and ammonia. A number of other organic bases were also tested. The results of these experiments are shown in Tables 4–6.

Table 4. *Penicillin production with different concentrations of starch and ethylamine*

Basal Medium but with 0.5 % glucose instead of 1 %.

| Starch<br>(%) | Ethylamine (%)              |     |      |
|---------------|-----------------------------|-----|------|
|               | 0.15                        | 0.3 | 0.45 |
|               | Titres (units/ml.) 10th day |     |      |
| 0             | 78                          | 56  | 38   |
| 1             | 81                          | 67  | 49   |
| 2             | 100                         | 105 | 72   |

With this basal medium the best results were clearly given by 2 % starch and not more than 0.3 % ethylamine. In another test with 2 % glucose and no acetic acid, the highest titre was 100 units/ml. with 0.45 % ethylamine and 1 % starch.

Table 5. *Effect of concentration of glucose and acetic acid on penicillin production with Basal Medium plus starch and ethylamine*

| Acetic<br>acid (%) | Glucose (%)                 |     |     |
|--------------------|-----------------------------|-----|-----|
|                    | 0.5                         | 1.0 | 2.0 |
|                    | Titres (units/ml.) 10th day |     |     |
| 0                  | 99                          | 68  | 19  |
| 0.25               | 85                          | 111 | 120 |

The results quoted in Table 5 show the effect of addition of glucose and acetic acid to Basal Medium containing starch and ethylamine. They were confirmed in repeat experiments, and should be compared with the results for



the same basal medium but without the starch and ethylamine. These showed that in the absence of starch and ethylamine penicillin production increased with glucose concentration whether acetic acid was present or not. Table 5, however, shows that when starch and ethylamine were present, increased concentration of glucose reduced production, unless acetic acid was present, in which case it increased it.

Table 6. *Effect of differing concentrations of starch, ethylamine and ammonia on yield of penicillin*

|                                       |      | Basal Medium |      |     |              |      |     |
|---------------------------------------|------|--------------|------|-----|--------------|------|-----|
|                                       |      | No starch    |      |     | 1.5 % starch |      |     |
| Ethylamine (%)                        |      | 0            | 0.15 | 0.3 | 0            | 0.15 | 0.3 |
| Titres (units/ml.) means of 8-10 days |      |              |      |     |              |      |     |
| NH <sub>3</sub> (%)                   | 0.6  | 38           | 85   | 87  | 25           | 64   | 84  |
| NH <sub>3</sub> (%)                   | 0.13 | 76           | 94   | 78  | 70           | 89   | 102 |

An examination of Table 6 reveals marked effects from the addition of ammonia and ethylamine and practically no effect from the addition of starch except with 0.3 % ethylamine and 0.13 % ammonia where the titre is markedly higher in the presence of starch.

When taken together the above results indicate that the media containing starch and ethylamine should give good yields in practice. This has, in fact, proved to be the case. With a simpler chemically defined medium, titres were usually about 75-90 units/ml. With the addition of starch and ethylamine, peak titres (10-11 days) were 134, 110, 105, 102, 113, 120, 103, 125 and 137, 112 and 113 units/ml. on different occasions. The mean is 119 units/ml., and compares favourably with the titres obtained with CSL media which usually reach 100-140 units/ml.

These results were all obtained with ethylamine. A number of other substances containing amino-groups has been tested. *n*-Butylamine and aspartic acid gave good results, ethanolamine gave poor results, and various other substances such as *isobutylamine*, glycine, alanine and propylamine were intermediate. Table 7 gives the results of one experiment with some of these compounds.

Table 7. *Effect on penicillin titres of addition of organic nitrogen to the Basal Medium (with 1.5 % starch)*

| Addition<br>(0.3 %)   | Penicillin<br>(units/ml. at 11 days) |
|-----------------------|--------------------------------------|
| Nil                   | 82                                   |
| Methylamine           | 79                                   |
| Ethylamine            | 106                                  |
| <i>n</i> -Propylamine | 100                                  |
| <i>n</i> -Butylamine  | 112                                  |
| <i>n</i> -Amylamine   | 58                                   |
| Dimethylamine         | 91                                   |
| Glutamic acid         | 113                                  |
| Aspartic acid         | 120                                  |
| Hydrolysed casein     | 105                                  |

## DISCUSSION

When the experiments were begun it was hoped to obtain chemically defined media which would give results comparable with those obtained with media containing CSL. The media which we have described resemble the latter in respect of the yield of penicillin and the macroscopic appearance of the mycelium they produce.

Suitable chemically defined media appear to require the simultaneous presence of several groups of components if the best results are to be obtained. Lactose is essential but is not satisfactory alone; glucose, or a similar carbohydrate such as glycerol, must also be added to ensure quick growth. Nor are these two carbon sources sufficient; the presence of an organic acid is also necessary in a fairly high concentration. For this we have found citric acid best, and a further addition of acetic acid is advantageous. The presence of a substance like phenylacetic acid, which enters the penicillin molecule directly, is also essential. It is also evident that the inorganic requirements must be carefully fulfilled if satisfactory results are to be obtained.

Table 8. *Comparison of corn-steep liquor and chemically defined medium*

| Important constituents             | CSL medium  | Chemically defined medium             |
|------------------------------------|---|---------------------------------------|
| (1) Principal carbohydrate sources | Lactose 3-4 %   | Lactose 3 %, glucose 1 %              |
| (2) Auxiliary carbohydrate source  | Non-reducing carbohydrate including polysaccharide    | Starch 1.5 %                          |
| (3) Organic acids                  | Acetic acid c. 0.05 %, lactic acid c. 0.5 %           | Acetic acid 0.25 %, citric acid 1.0 % |
| (4) Special compounds              | $\beta$ -Phenylethylamine and hydroxyphenylethylamine | Phenylacetic acid 0.05 %              |
| (5) Principal nitrogen sources     | Mixture of amino-acids, amines and ammonia            | Ammonium sulphate 0.5 %               |
| (6) Secondary nitrogen source      |   | Ethylamine 0.3 %                      |
| (7) Total solutes                  | 8-9 %   | c. 8.5 %                              |
| (8) Total nitrogen                 | 0.15-0.20 %   | c. 0.20 %                             |

In certain respects the best chemically defined media show a marked similarity of composition to CSL media. The groups of ingredients which seem to be essential, and the substances representing them in CSL and in the chemically defined media, are shown in Table 8. It appears that each group of components in the one medium has its counterpart in the other. Nitrate is often added to both media, and may increase the yields slightly.

Media prepared on the above lines can, in absence of starch and ethylamine, be expected to yield 70-90 units/ml. of penicillin. The addition of starch or certain dextrans together with short-chain aliphatic amines such as ethylamine increased the penicillin yield to an average of 119 units/ml.; this is of the

same order as the yields given by media containing CSL under our conditions of growth.

The reasons for the specific advantages of each component of the medium are not known. It seems possible that lactose and starch act by reason of their slow assimilation which is perhaps dependent upon a prior hydrolysis. The other nutrients, such as acetic acid, citric acid, amino-acids and primary amines, may act as carbon sources or assist in the uptake of nitrogen or affect the regulation of pH.

It is noteworthy that there is little or no interaction between the various substances in the media: the introduction of each constituent, citric acid, acetic acid, ethylamine, etc., usually produced an increase in titre, the effect of each being additive. The only exception so far encountered lies in the effect of adding acetic acid and glucose in the presence or absence of ethylamine and starch together. In the absence of the last two, penicillin yield is about the same with increased addition of glucose, with or without acetic acid. In presence of ethylamine and starch, however, increase of glucose depresses production of penicillin unless acetic acid is present at the same time, in which instance it increases it. There is no obvious explanation of this effect, but it suggests that as the media become more effective and more complex, the interrelation between their components becomes more critical.

The new media and CSL media both have a high content of total solutes. They have also about the same concentration of nitrogen. In addition to these similarities, the complex nature of the new media serve to illustrate the convenience conferred by using CSL. In so far as one can base any hypotheses on the above results, it would appear that CSL owes its particular suitability for penicillin production to the fortuitous presence therein of several classes of inorganic and organic substances necessary for penicillin production by *P. notatum* rather than to the presence of a particular essential substance. Our experiments show that the effects observed with CSL can also be produced by means of suitable chemically defined mixtures. They do not, however, exclude the possibility that CSL may owe some of its usefulness to an unidentified nutrient or growth factor.

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## Development of Bacteria in Waters Stored in Glass Containers

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**SUMMARY:** The factors affecting the growth of bacteria in fresh water stored in different containers, usually glass, were studied in order to reconcile the different results which have been obtained by previous workers. Growth occurred in two sites—in the body of the water and at the surface of the container—and was affected by the constituents of the container.

Bacteria invariably grew on the sides of the container and were presumably dependent for their multiplication on having a site of attachment; thus the increase in the count per unit volume which occurred when bottles were vigorously shaken was greater in small bottles than in large bottles, and was due to removal of some of the cells attached to the walls. Bacterial growth was stimulated by soluble chemical substances in the walls of containers; Bohemian glass and the glass of measuring cylinders were stimulatory; Pyrex glass and fused silica containers were inactive. It is probable that under the conditions of their experiments some workers have been observing bacteria which were dependent on the glass surface for their existence and which were unable to multiply in the body of the water sampled.

Since the publications of the Franklands (1894) and Whipple (1901) it has become well established that when fresh water or sea water is stored in glass containers bacteria multiply rapidly to numbers which are often far in excess of those found under natural conditions. But although it is agreed that the phenomenon takes place the cause remains a matter for discussion. The subject has been reviewed in detail by ZoBell & Grant (1943) and by ZoBell (1946). It has usually been found that the number of bacteria developing in a glass container is related to its size, appreciably greater numbers being found in waters stored in small vessels than in large; but Castell & McDermott (1941) found no such relationship. Lloyd's (1937) tests were made in narrow-mouthed cylindrical Pyrex jars of capacities 100, 1000 and 8000 ml., and the plate count from the 100 ml. jar was approximately three times those of the samples from the 1000 and 8000 ml. jars, which did not differ significantly. The phenomenon was not dependent on the ratio of surface area of the liquid to volume. ZoBell & Anderson (1936) concluded that the development of a denser population in the smaller vessel was due to the greater ratio of surface area of the container to volume and to the ability of certain types of bacteria to multiply on a glass surface. ZoBell (1943) later suggested that the nutrient organic matter from the water is concentrated by adsorption on the glass surface, and that solid surfaces retard the diffusion of exoenzymes and hydrolysates from the cell. This hypothesis of adsorption of organic matter received some support from Stark, Stadler & McCoy (1938), and from Harvey (1941). It is based first on the well-established fact that on glass surfaces in contact with natural waters sooner or later develops there an attached film of bacteria which can be demonstrated by staining; secondly, on claims that chemical methods can detect and measure

the matter adsorbed on the glass surface. However, Harvey (1941) was unable to demonstrate significant amounts of organic matter by the alkaline potassium permanganate method, and the often-quoted paper by Stark *et al.* (1938) gives neither details of the bichromate method used, nor any quantitative data.

The phenomenon of growth of bacteria in stored waters is of more than academic interest; it is an example of the growth of bacteria in extremely dilute solutions. The hypotheses of adsorption and consequent concentration of organic matter on the glass sides of the container from the stored water, and the enzyme concentration theory of ZoBell (1943) are not wholly consistent. On the one hand the growth of bacteria in waters transferred to containers is rapid, and maximum numbers are usually found on the second or third day after filling; on the other hand, the development of attached bacteria is much slower, and appreciable numbers, as demonstrated by direct methods, do not occur until some time later (ZoBell & Stadler, 1940, Fig. 2). With regard to organic matter on the glass surface the published results of chemical tests are quite inadequate to prove its existence. Moreover, any organic matter present might be bacterial protoplasm and not due to adsorption of soluble compounds from the water. Furthermore, chemically clean dry glass is capable of reducing potassium permanganate in the absence of organic matter (Taylor, 1947), so that past experiments using glass beads and glass tubes might have been falsely interpreted. The need for controls in this type of work is obvious.

*Methods.* Except where otherwise stated, samples of water were collected in Winchester quart bottles and brought to the laboratory within 1 hr. of collection. Samples from Lake Windermere were taken at a depth of approximately 1 m. in mid-lake. The samples were dispensed into the various experimental containers and, unless otherwise stated, incubated at a temperature of 20°. Plate counts were made in peptone casein starch glycerol medium (Taylor, 1940) employing 3-5 replicate Petri plates/sample. The plates were incubated for 12 days at a temperature of 20°.

## EXPERIMENTAL

### *Development of bacteria in containers*

*Multiplication in lake water: the effect of temperature.* Plate counts were made at 2 hr. intervals for a period of 26 hr. from two 500 ml. samples of Windermere water contained in Winchester bottles; one bottle was incubated at room temperature (17.2-20°) and the other at 20°. Both containers were agitated vigorously before removing the samples. The counts in both samples were stationary during the first 12 hr. From the 12th hour onwards counts increased in both samples but more rapidly in that kept at the slightly higher temperature. At 26 hr. the counts were 130,000/ml. in the 20° sample and 14,800/ml. in the 17.2-20° sample (Fig. 1).

*The effects of the material of the container on multiplication of bacteria.* Three tests were carried out with Windermere water in containers of different material. In the first test 500 ml. samples were held in (a) 1 l. Pyrex conical flasks, (b) 1 l. flint glass bottles and (c) 1 l. tall bottles made of gutta percha.

In the second test 1 l. conical flasks of Pyrex, Bohemian glass, and of fused silica ('Vitreosil') of almost identical shape were employed. In both experiments three replicate containers of each kind were used, and the figures given in

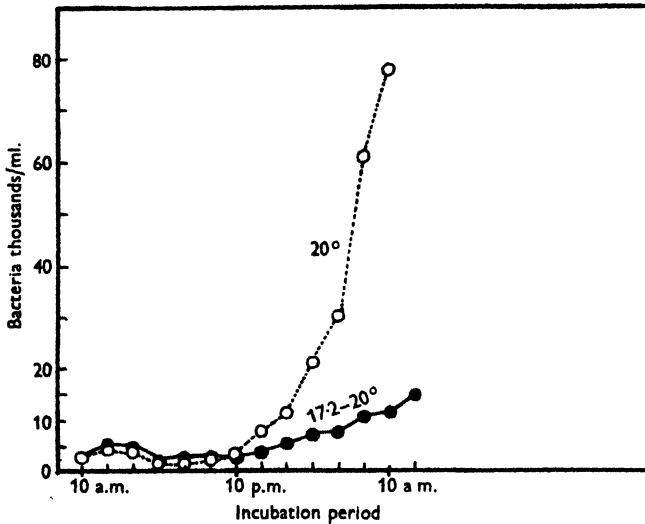


Fig. 1. Plate counts of bacteria at 2 hr. intervals in Windermere water incubated at 20° and room temperature (17.2-20°) in 1 l. Winchester bottles, with vigorous shaking on each sampling occasion. ○---○ 20°; ●—● 17.2-20°.

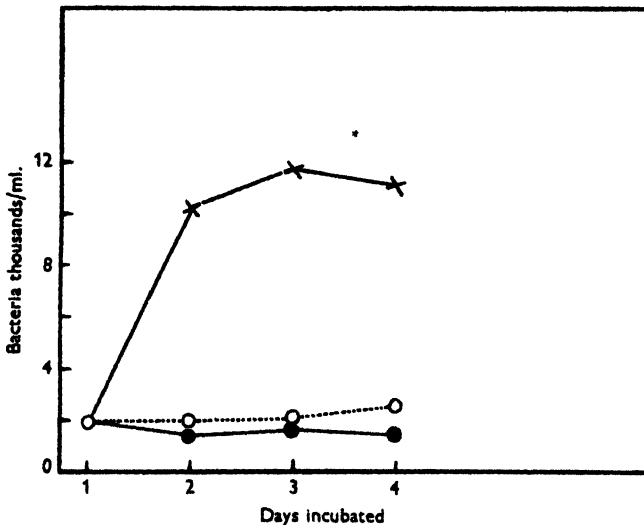


Fig. 2. Plate counts of bacteria in Windermere water incubated in 1 l. conical flasks of different chemical composition for 4 days at 20°. x—x Bohemian glass; ○---○ Pyrex glass; ●—● fused silica.

Table 1 and Fig. 2 represent the average counts obtained from these replicates. The containers and their contents were not agitated, and samples were pipetted from approximately 2.5 cm. below the surface. The results show that

under quiescent conditions no significant increase in numbers of bacteria took place in the water stored in the Pyrex, flint, or silica containers, but a marked increase in the gutta percha and Bohemian glass containers.

Table 1. *Plate counts of bacteria in Windermere water stored in (a) 1 l. Pyrex conical flasks, (b) 32 oz. flint glass bottles and (c) in 1 l. gutta percha stoppered containers for 4 days at 20°*

| Counts (thousands/ml.)   |              |                |                         |
|--------------------------|--------------|----------------|-------------------------|
| Incubation period (days) | Pyrex flasks | 32 oz. bottles | Gutta percha containers |
| 0                        | 3.4          | 3.4            | 3.4                     |
| 1                        | 6.1          | 5.5            | 4.4                     |
| 2                        | 11.0         | 10.0           | 129.0                   |
| 3                        | 10.0         | 10.0           | 97.0                    |

In a third test three replicate Winchester quart (1 l.) bottles were compared with 1 l. Pyrex conical flasks, each containing 500 ml. of Windermere water, the water being agitated by rotation of the container before sampling. The results (Table 2) show the enhanced bacterial count in the water in the Winchester bottles.

Table 2. *Plate counts of bacteria in replicate samples of Windermere water incubated in Winchester bottles and in Pyrex flasks of 1 l. capacity*

| Incubation period (days) | 1 l. Winchester bottles |     |     |     | 1 l. Pyrex flasks      |    |    |     |
|--------------------------|-------------------------|-----|-----|-----|------------------------|----|----|-----|
|                          | 1                       | 2   | 3   | Av. | 1                      | 2  | 3  | Av. |
|                          | Counts (thousands/ml.)  |     |     |     | Counts (thousands/ml.) |    |    |     |
| 0                        | 16                      | 16  | 16  | 16  | 16                     | 16 | 16 | 16  |
| 1                        | 142                     | 260 | 44  | 148 | 22                     | 44 | 26 | 31  |
| 2                        | 227                     | 250 | 122 | 200 | 52                     | 50 | 42 | 48  |
| 3                        | 252                     | 235 | 125 | 205 | 5                      | 10 | 8  | 8   |

The results of the three experiments indicate that when Windermere water was stored in containers made of relatively insoluble materials no significant multiplication of bacteria took place. When, however, the container was agitated so as to remove bacteria developing on the glass surface the bacterial count increased, and this increase was greater in the containers made of soft glass (Winchesters) than in those made of resistance glass. Consequently, it was decided to determine whether the apparent multiplication of bacteria in stored water was connected with the growth of bacteria on the glass surface.

*The effect of agitation.* To ascertain whether bacterial growth in stored samples of water might not take place partly, or entirely, on the walls of the container, samples were taken from water (a) kept under quiescent conditions and (b) after vigorous agitation. Five hundred ml. amounts of Windermere water were stored in 1 l. Pyrex flasks, and on each sampling occasion three flasks were treated each as follows: (1) not agitated; (2) shaken vigorously before insertion of pipette, and after withdrawing sample returned to incubator;



(8) shaken vigorously and discarded after sample had been taken. The results (Table 8) showed that although counts of bacteria increased in all samples, there were appreciably larger counts in the samples taken after agitation. It thus appears that dislodgement of bacteria attached to the sides of the container by agitation was an appreciable factor in the apparent multiplication of bacteria in stored water.

Table 8. *Plate counts of bacteria in Windermere water incubated in 1 l. Pyrex conical flasks for 4 days at 20° (a) under quiescent conditions, (b) shaken on each sampling occasion and (c) shaken once only*

| Incubation<br>period (days) | Counts (thousands/ml.) |             |              |
|-----------------------------|------------------------|-------------|--------------|
|                             | Quiescent              | Shaken once | Shaken daily |
| 0                           | 2.1                    | 2.1         | 2.1          |
| 1                           | 5.2                    | 5.4         | 4.1          |
| 2                           | 7.8                    | 8.6         | 15.8         |
| 3                           | 5.2                    | 18.2        | 18.2         |
| 4                           | 4.7                    | 23.4        | 14.6         |

*The effect of the size of container.* Triplicate sets of Pyrex flasks of capacities 150, 500 and 2000 ml. were filled to half-capacity with Windermere water and incubated under quiescent conditions. Very little increase in plate count took place, and the size of the container played no significant part (Table 4). A similar experiment with water from a polluted well showed (Fig. 3) that though bacteria grew appreciably in all containers the counts in different-sized containers did not differ significantly.

Table 4. *Plate counts of bacteria in Windermere water incubated in Pyrex conical flasks of different capacities for 3 days at 20°*

| Incubation<br>period (days) | Flask capacity (ml.)   |     |      |
|-----------------------------|------------------------|-----|------|
|                             | 150                    | 500 | 2000 |
|                             | Counts (thousands/ml.) |     |      |
| 0                           | 1.5                    | 1.5 | 1.5  |
| 1                           | 2.6                    | 3.3 | 1.6  |
| 2                           | 2.0                    | 3.2 | 1.8  |
| 3                           | 3.0                    | 4.6 | 2.0  |

In a third experiment carried out in duplicate, bottles of 50, 75, 150, 250, 500 and 1500 ml. capacity were filled to approximately half-capacity with Windermere water, and agitated by vigorous rotational movements before sampling. The results with duplicate bottles of the smaller sizes (50 and 75 ml. amounts) were rather discrepant, but in general the bacteria multiplied more rapidly and to greater numbers in the smaller bottles (Table 5). As the bottles were not of the same brand and therefore not necessarily of the same chemical composition, the possible variation due to differing solubilities of the constituents cannot be entirely disregarded.

In a fourth experiment two sizes of Pyrex flasks, 150 and 1000 ml., were

half-filled with polluted well water. On each sampling occasion two flasks of each size were agitated vigorously, sampled and returned to the incubator, and two flasks of each size which had not been sampled previously were similarly treated but discarded after sampling. The results showed (Fig. 4) that appreciably greater numbers of bacteria developed in the smaller flasks than in the larger, and that repeated agitation resulted in greater numbers than a single agitation; a result not in agreement with the finding with Windermere water.

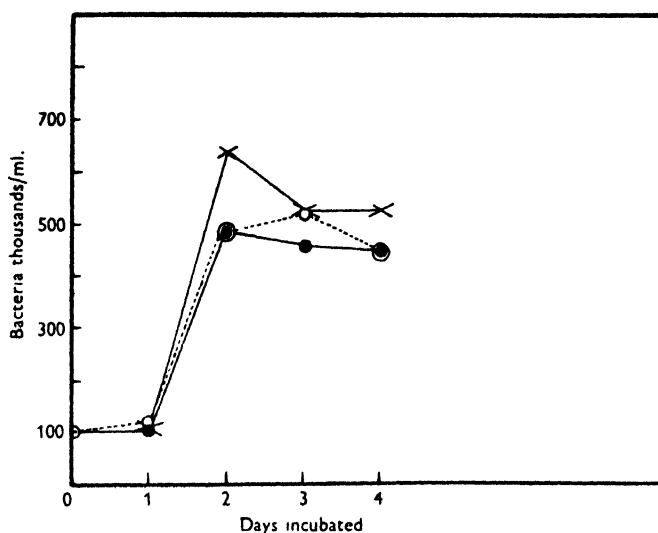


Fig. 3. Plate counts of bacteria in water from a polluted well incubated in Pyrex conical flasks of different capacities for 4 days at 20°. x — x 150 ml.; o — o 500 ml.; ● — ● 2000 ml.

Table 5. Plate counts of bacteria in Windermere water incubated in bottles of different capacities for 3 days at 20° and shaken before sampling

| Incubation period (days) | Bottle capacity (ml.)  |       |       |      |      |      |
|--------------------------|------------------------|-------|-------|------|------|------|
|                          | 50                     | 75    | 150   | 250  | 500  | 1500 |
|                          | Counts (thousands/ml.) |       |       |      |      |      |
| 0                        | 2.2                    | 2.2   | 2.2   | 2.2  | 2.2  | 2.2  |
| 1                        | 6.2                    | 3.1   | 2.1   | 3.7  | 3.0  | 3.3  |
| 2                        | 79.3                   | 75.0  | 43.6  | 10.6 | 24.6 | 12.0 |
| 3                        | 76.2                   | 110.3 | 119.4 | 62.2 | 75.0 | 69.3 |

In a fifth experiment triplicate sets of measuring cylinders of 25 and 1000 ml. capacities were filled with Windermere water. The water was allowed to remain undisturbed during incubation, and samples were withdrawn daily by means of a pipette; no contact of the pipette with the sides of the cylinders took place. The results (Table 6) were quite spectacular, for whereas the count in the water stored in the 1000 ml. cylinder increased from 2000 to a maximum of 12,200/ml., the count in the 25 ml. cylinder increased to a maximum

of 244,000/ml., the highest figure ever recorded for Windermere water. This enormous difference in numbers of bacteria in the large and small cylinders bears no simple relationship to the small difference in the ratios of volume to surface area of the containers (ml./cm.<sup>2</sup>), which was 10.8 and 8.5 respectively.

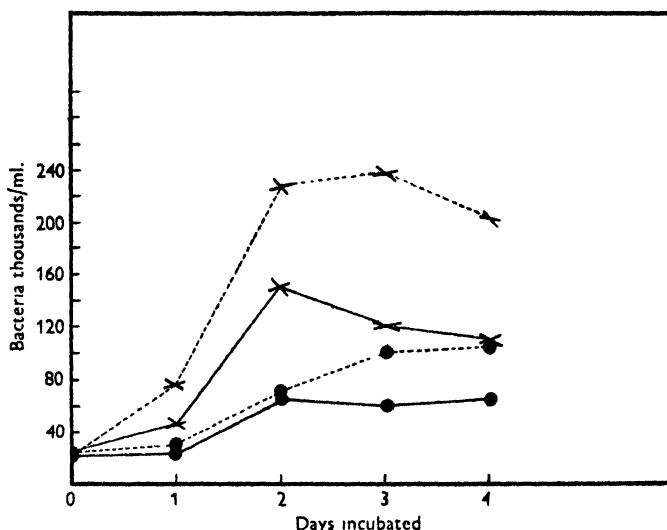


Fig. 4. Plate counts of bacteria in water from a polluted well incubated in 150 and 1000 ml. Pyrex conical flasks for 4 days at 20°. One set of flasks was shaken on each occasion and one set shaken only at the time of sampling and subsequently discarded. x---x 150 ml. shaken each time; x—x 150 ml. shaken once; ●---● 1000 ml. shaken each time; ●—● 1000 ml. shaken once.

Table 6. Plate counts of bacteria in Windermere water incubated under quiescent conditions in glass measuring cylinders for 4 days at 20°

| Incubation period (days) | Cylinder capacity (ml.) |      |
|--------------------------|-------------------------|------|
|                          | 25                      | 1000 |
|                          | Counts (thousands/ml.)  |      |
| 0                        | 2.0                     | 2.0  |
| 1                        | 6.8                     | 8.4  |
| 2                        | 248.6                   | 10.6 |
| 3                        | 115.0                   | 8.6  |
| 4                        | 142.2                   | 12.2 |

#### Chemical estimation of organic matter

*Oxygen absorbed from acidified KMnO<sub>4</sub>.* In view of the claims (ZoBell, 1948) that organic matter is adsorbed from water on to the glass surface of a bottle, and can be demonstrated by chemical analyses using the permanganate or bichromate reduction tests, we have made extensive tests with 0.0125 N-KMnO<sub>4</sub> acidified by 25 % (v/v) H<sub>2</sub>SO<sub>4</sub>. Boiling the reagents and sample for a specified time gave unsatisfactory and unreproducible results, but the method commonly

used in Britain of incubation for 4 hr. at a temperature of 80° F. (26·7°C.) gave reproducible results. Taylor (1947) showed that  $\text{KMnO}_4$  in distilled water in the presence of a dried glass surface was appreciably and inexplicably reduced. When the glass surface was increased by adding glass balls, the amount of oxygen absorbed from the acid permanganate in the absence of organic matter was as great or greater than the oxygen absorbed by permanganate from water taken from Windermere and other local sources. Consequently, ZoBell's (1943) experiments with glass beads, glass-wool, and glass tubes, cannot be interpreted as measuring organic matter only; they need repeating with adequate controls.

Table 7. *Oxygen absorbed from acid permanganate (p.p.m.) from waters (1) freshly gathered, (2) after storage at 20° for 16 days and (3) by the surface of the container after a similar period*

|                          | Start | 16 days | Difference | Glass surface |
|--------------------------|-------|---------|------------|---------------|
| Blelham Tarn             | 1·84  | 1·84    | 0·00       | 0·12          |
| Inflow I                 | 2·18  | 1·94    | -0·24      | 0·29          |
| Inflow II                | 2·04  | 1·87    | -0·17      | 0·36          |
| Inflow III               | 0·64  | 0·54    | -0·10      | 0·22          |
| Windermere               | 0·78  | 0·70    | -0·08      | 0·00          |
| Windermere + glass beads | 1·28  | 1·21    | -0·07      | 0·38          |

Measured amounts of water (100 ml.) were placed in bottles, and the oxygen absorbed from acidified  $\text{KMnO}_4$  was determined (1) at once, (2) after periods of incubation at 20°, (3) after the same periods but with careful decantation of the water and replacement with an equal volume of freshly distilled water. The results of a typical experiment are shown in Table 7.

It is evident that the organic-matter content of the water decreased during storage, particularly that of waters flowing into Blelham Tarn, but the amounts were extremely small and there was nothing to suggest that the glass surface, particularly that of 150 g. glass beads of 2–3 mm. diameter, favoured the decomposition of the organic matter. On the other hand, when the surface of the glass was tested for organic matter it could be detected with all samples except that of Windermere water. In a repeated experiment with water from Windermere, Blelham Tarn and a well, tests for organic matter were negative.

*Oxygen consumed (biochemical oxygen demand).* If organic matter is adsorbed on the glass surface and is therefore more readily available for bacterial decomposition, the oxygen demand of water stored in small vessels should be greater than that of water stored in large vessels, owing to the relatively greater surface of glass available in the smaller container. According to ZoBell & Anderson (1936) this happens with stored sea water; but with lake water, ZoBell & Stadler (1940) found no significant difference between the amounts of oxygen consumed in samples incubated in 145 and 4000 ml. amounts. We have made similar tests with water from local lakes in a range of bottles of different sizes, making allowances for the volumes of reagents to be added. A constant-temperature water-bath at 20° was used for incubation, and sets

of three bottles were analyzed on each occasion. The results exemplified in Table 8 show that with lake water the size of the container does not affect the amount of organic matter decomposed by bacteria. The organic matter in these waters is so resistant to bacterial attack and so slowly broken down that its concentration is unlikely to influence its rate of decomposition. It is, however, possible that in very dilute solutions of sewage effluents, in which the organic matter was readily available, its concentration might influence the total amount decomposed or the rate of decomposition.

Table 8. *Biochemical oxygen demand (p.p.m.) of water from Blelham Tarn incubated in bottles of different capacities at 20° for different periods*

| Capacity of<br>bottle (ml.) | Days incubated                     |      |      |
|-----------------------------|------------------------------------|------|------|
|                             | 2                                  | 5    | 10   |
|                             | Biochemical oxygen demand (p.p.m.) |      |      |
| 70                          | 0.49                               | 0.85 | 1.15 |
| 140                         | 0.49                               | 0.86 | 1.26 |
| 250                         | 0.45                               | 0.87 | 1.29 |
| 400                         | 0.43                               | 0.88 | 1.15 |
| 140                         | —                                  | 0.29 | 0.52 |
| 2700                        | —                                  | 0.32 | 0.51 |

## DISCUSSION

In this study two facts can be accepted as well established. First, when waters, including distilled water, are stored in glass containers and sampled from time to time by a bacteriological routine including shaking the container, the bacteria appear to multiply to numbers greatly in excess of those found in the water under natural conditions. Secondly, low storage temperatures repress multiplication and raised temperatures usually increase it. Before considering our results it would be well to consider the changes which must result in water from the sea, lakes, rivers or wells when it is stored in comparatively small containers. In the first place natural circulation ceases and some particulate matter settles to the bottom of the container; any planktonic animals die, and if the container is stored in darkness, algal cells also perish. In addition, the solid surfaces of the walls of the container are available for the attachment of periphytic types of bacteria. If we assume that phosphorus and other essential inorganic ions are present, as is almost invariably the case, the water is an extremely dilute medium for the growth of bacteria, and the degree of growth will depend on the supplies of nitrogen, carbon and sources of energy. To some extent these materials are provided by dead planktonic cells. There is evidence also that whereas the freshly sampled water may contain a great variety of bacteria, storage conditions act selectively and encourage only a small proportion of the types originally present. Thus the dead cells of the types unable to grow may provide further nourishment for the selected types.

Our experiments show that when samples of water are stored in containers of resistance glass or silica the numbers of bacteria found are invariably greater

when the container is shaken before sampling than when quiescent conditions are maintained; under quiescent conditions there is often no significant increase in numbers of bacteria. The agitation of the samples removes from the sides of the container those types of bacteria which are dependent for their existence on the presence of a solid surface and which under natural conditions are dependent on solid particles in suspension. Under such quiescent conditions of storage the bacterial numbers are similar to those in natural conditions.

The effect of the composition of the container is no less striking. No material is completely insoluble in the presence of water, and it is well known that soft glass is far more soluble than resistance glass. The evidence suggests strongly that water-soluble substances are here stimulating bacterial activity. A comparison of the chemical composition of soft and resistance glass suggests that sodium, potassium, calcium, magnesium or sulphate ions might be responsible agents.

Workers, in general, have given little information about the amount their experimental containers were shaken before sampling, or about their composition; and the discordant results reported may be due in part to variations in technique in this regard. Castell & McDermott (1941) were perplexed that a well water having an average bacterial count of less than 10/ml. when stored for 4 days yielded a count of *c.* 1 million/ml.; in this instance conditions were more complex. Their chemical analyses suggest that the water contained an appreciable amount of organic matter (volatile soluble substances 100 p.p.m.), and an exceptional amount of magnesium (250 p.p.m.). A very varied collection of containers was used in the experiment, but probably the most significant point was the increase in temperature from that of the well (unstated) to room temperature (25°), at which the samples were incubated.

ZoBell's hypothesis of the adsorption of organic matter on the walls of the container has not been confirmed; and it cannot be entirely refuted. Determinations of oxygen consumed and oxygen absorbed from acid permanganate show that organic matter in the water has been broken down, but we have no evidence that an increase in surface area in relation to the volume of the container increases the amount of organic matter decomposed, as would be expected if ZoBell's theory were correct. On the other hand, bacteriological tests indicate the growth of bacteria on the walls of the container, and, with some waters, organic matter is demonstrable on the sides of the container. On the available evidence we conclude that with lake and tarn waters such organic matter as may develop on the sides of a container is due to that of bacterial cells. Such a conclusion would reconcile the results of tests for oxygen consumed in bottles of different sizes with tests for organic matter adsorbed on the glass surface.

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## A Note on Morphological Differences between Strains of *Streptococcus cremoris*

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**SUMMARY:** Some strains of *Streptococcus cremoris* are slightly more heat resistant than the majority. After growth in milk at 37° for 5 hr. some of them show under the microscope involution forms which are sufficiently characteristic to serve as a useful means of identification.

A number of strains of *Streptococcus cremoris* peculiarly suited for use as 'starters' in Cheddar cheese manufacture were selected from hundreds isolated during the past 12 years. The strains were selected for:

- (1) great activity in the fermentation of lactose in milk culture and in cheese curd;
- (2) power to withstand an incubation temperature of 37° without suffering as much damage as most strains suffer;
- (3) total absence of any relation to each other in their reactions to a series of bacteriophages.

The cultures have been regularly plated and repurified at intervals of about 2 months (some of them over seventy times), so that their characters are likely to be as stable as possible. There is evidence, however, that during the period of observation the rate of acid production decreased slightly in some of them. Since all the strains belong to one species there are not many features by which they may with certainty be distinguished. There are, however, some apparently characteristic morphological differences between them which have proved exceedingly valuable, not only in distinguishing these particular strains but also in enabling us to predict whether a freshly isolated unknown strain is related to any of them.

### *The demonstration of morphological differences*

The optimum temperature for growth of most strains of *Strep. cremoris* in milk is in the region of 30°; at 37° the organisms still grow but are somewhat inhibited. Subjected to the latter rather unfavourable temperature the various strains exhibit differences in morphology which have a value in identification. The stock strains when grown in skim milk at 22° or 30° cannot be distinguished from one another under the microscope at any stage of growth.

Figs. 1-3, Pl. 1, illustrate the appearance of the three strains, HP, K and R 6 after incubation for 5 hr. at 30°. All the strains form the long chains of spherical cocci typical of *Strep. cremoris*. When, however, the organisms are incubated in milk culture at 37° for 5 hr. they assume forms which are characteristic enough to serve as identifying features. This is illustrated in Figs. 5-7 (Pl. 1), where the following points may be noted.



**Culture HP.** Cocci still spherical, but terminal cocci in many chains are swollen to about twice normal size (Fig. 5).

**Culture K.** Cocci have assumed flat-sided squarish shape, and the chains have a characteristic tangled arrangement (Fig. 6).

**Culture R<sub>8</sub>.** Very long chains of flattened cocci crushed together concertina-fashion (Fig. 7).

All the cultures grow less at 37° than at 30°, a reflexion of the unsuitability of 37° as a growth temperature for *Strep. cremoris*.

The involution shapes of several other strains of *Strep. cremoris* are similarly quite characteristic, and on many occasions served as a means of predicting that an unknown culture was related to one of the stock strains. The three strains described can be identified with certainty by the method described. Other strains show less definite changes in morphology which, nevertheless, are capable of recognition by the experienced worker; still other active strains cannot be positively identified by this means. A large number of strains of *Strep. cremoris* which are not active enough to be used as cheese 'starters' are much more heat sensitive and react to incubation at 37° as shown in Figs. 4 and 8 (Pl. 1). It will be observed that whereas this strain shows a healthy growth at 30°, it is very feeble and badly involuted at 37°.

#### *Distinction between Strep. cremoris and Strep. lactis*

Although many workers still do not agree that *Strep. cremoris* is distinct as a species from *Strep. lactis* we find that most *lactis* types which ferment maltose and dextrin and which form only diplococci or very short chains in culture, also give quite a different appearance from our stock *cremoris* strains after growth at 37°. They are not significantly inhibited even at 37°, and the cocci (smaller even at 30° than those of *Strep. cremoris*) are not significantly altered in shape or arrangement at the higher\* temperature. This is in accord with expectation, since strains of *Strep. lactis* have in general a higher optimum and maximum growth-temperature than strains of *Strep. cremoris*. Thus it is usually possible on the basis of morphology at different incubation temperatures to divide lactic streptococci into groups which correspond with Orla-Jensen's species *Strep. lactis* and *Strep. cremoris*, and these groups are also characterized respectively by ability and inability to ferment maltose and by a different pattern of sensitivity to various phages (Hunter, 1946). There is, however, a small proportion of intermediate types which cannot be definitely assigned to either species.

#### DISCUSSION

The characteristic morphology assumed by many strains of *Strep. cremoris* after incubation in milk at 37° for 5 hr. is a very useful criterion, both for selecting active and heat-resistant cultures and for predicting the phage relationships between strains. In the course of a search extending over several years for unrelated strains of *Strep. cremoris* we have gained the impression that the number of types is relatively limited. Thus only seven unrelated strains



Figs. 1-8



active enough for our purpose have been found over the course of about 10 years. It is, however, possible that within any one country a small group of types becomes widespread, with the result that the same strains are constantly being encountered. There are some indications that unrelated strains are more easily found in material from abroad. We hope to investigate this possibility further.

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#### EXPLANATION OF PLATE

(Magnification  $\times 900$ )

Figs. 1-3. *Strep. cremoris* cultures HP, K and R<sub>4</sub> after 5 hr. growth at 30°.

Figs. 5-7. The same cultures after 5 hr. growth at 37°.

Figs. 4, 8. A heat-sensitive strain of *Strep. cremoris* after growth at 30 and 37° respectively for 5 hr.

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## The Isolation and Cultivation of Sulphate-Reducing Bacteria

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**SUMMARY:** There are many strains and perhaps several species of sulphate-reducing bacteria. They may be isolated by using a variety of media over a wide range of temperature. Crude cultures are readily obtained, but isolation of absolutely pure cultures is usually difficult. Considerable simplification in procedure may sometimes be effected by including 3%  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  in the media; the sulphite eliminates most of the contaminating organisms in crude cultures and facilitates subsequent purification. A method of maintaining stock cultures on sterile clay is preferred to the use of artificial media.

No growth takes place in media rendered 'biologically free' of iron, but the traces normally present in media constituents as impurities are sufficient for good development. Rapid and abundant growth free from ferrous sulphide has been obtained in a mineral salts + lactate + yeast extract medium containing ferrous ions below the saturation concentration for ferrous sulphide.

One thermophilic and four mesophilic strains have been shown to be facultative autotrophs, using the oxidation of hydrogen as energy source, with sulphate, sulphite, thiosulphate and elementary sulphur as hydrogen acceptors. No autotrophic growth takes place in the absence of hydrogen. Hydrogen can be provided *in vitro* by immersing metallic iron in the medium.

Although 'sulphate reduction is one of the most commonly occurring and extensive microbiological processes on earth' (von Wolzogen Kühr & van der Vlugt, 1984), sulphate-reducing bacteria are physiologically unique among living organisms in being able to reduce sulphates to sulphides; Tanner's (1918) suggestion that some yeasts possess this property has not been confirmed (Bunker, 1936). They are also capable of reducing sulphites, thiosulphates and elementary sulphur to sulphides. Practically every type of soil and natural water contains them and they are widely distributed in the sea. Holland, a country of odoriferous canals and great microbiologists, contributed most to our early knowledge of them. First described by Beijerinck (1895) and isolated in pure culture by van Delden (1903), their physiology and biochemistry was comprehensively investigated by Baars (1930), while von Wolzogen Kühr & van der Vlugt (1984) defined their function as essential agents in the anaerobic corrosion of buried ferrous pipes. The ubiquity of the organisms led inevitably to their investigation in other countries (see Tausson & Alioschina, 1982; Copenhagen, 1984; Bunker, 1936, 1988; Starkey, 1988; Datta, 1948; ZoBell, 1946a, 1947).

Sulphate-reducers play an important part in the sulphur cycle in nature, both on land (Bunker, 1936) and in the sea (ZoBell, 1946a). Their activities, chiefly because invariably accompanied by the production of hydrogen sulphide, frequently result in undesirable manifestations, sometimes of considerable economic importance, as in the corrosion of buried metal structures or the

contamination of coal, gas and oil. They are said to contribute to the formation of petroleum and to its modification after formation (ZoBell, 1945). A recently discovered and potentially valuable property of some strains is their power of releasing oil from oil-bearing sediments (ZoBell, 1947).

During our work on anaerobic microbiological corrosion we have been faced with the many difficulties of obtaining pure cultures of these organisms in quantities sufficient for biochemical investigation. We describe below improved methods of isolation and cultivation, demonstrate the autotrophic growth of several pure strains, and discuss related questions of nomenclature and classification.

### *Nomenclature and classification*

The nomenclature of sulphate-reducing bacteria is confusing and their classification still uncertain. Since their first description by Beijerinck (1895) it has been generally agreed that there is only one genus, variously designated *Bacillus*, *Bacterium*, *Spirillum*, *Microspira*, *Vibrio*, *Sporovibrio* and *Desulphovibrio*. Kluver & van Niel (1936) placed them in the tribe *Vibrionae* with the generic name *Desulphovibrio*. In Bergey's *Manual*, 5th edition (Bergey, 1939), they were included in the tribe Spirillaceae, genus *Spirillum*, in an appendix containing the 'additional species (which) have been mentioned in the literature'. In the 6th edition of Bergey (1948) they are retained in the tribe Spirillaceae (family Pseudomonadaceae), but are given the generic name *Desulphovibrio*.

The many species-names include *hydrosulphureum*, *hydrosulphureus*, *desulphuricans*, *thermodesulphuricans*, with *aestuarii* and *rubentschikii* for halophilic strains. ZoBell (1947) has recently added *hydrocarbonoclasticus* for a species attacking petroleum hydrocarbons and *halohydrocarbonoclasticus* for the corresponding halophilic variety.

It may provisionally be agreed that *Desulphovibrio* should be the generic designation of sulphate-reducing bacteria. The question of species names is more complicated. Until 1980 it was thought there were three species, i.e. non-halophilic, halophilic and thermophilic. Baars (1980), however, showed that these three supposed species were interconvertible by gradual acclimatization, and he regarded them as three strains of a single species, *Vibrio desulphuricans*. Since then many new varieties have been isolated. Bunker (unpublished observation) was unable to induce growth of some of his strains at temperatures higher than 37°. Rittenberg (1941) isolated obligate mesophilic and halophilic varieties from marine sediments. Sporing cultures have been described by Starkey (1938), but not all sulphate-reducers can be induced to form spores. All strains so far reported in the literature are motile, but we possess a culture isolated by Bunker in this laboratory from Thames river mud which is non-motile and shows no flagella in electron micrographs. ZoBell (1947) described strains which attacked hydrocarbons, a property not possessed by all sulphate-reducers; he also described special strains which grew at 80°. Although some of these differences would not in themselves justify the creation of separate species, there appears to be sufficient evidence for assuming the existence of several species. In the choice of names for new species we suggest adherence

to Recommendation 6b (2) of the International Bacteriological Code of Nomenclature, 1947, i.e. 'to avoid those which are very long and difficult to pronounce'. We recommend that species names of more than five syllables should be avoided.

The variety of sulphate-reducing organisms has some bearing on the isolation of pure cultures. It is not sufficient to develop cultures from any given source on one medium alone. Media suitable for both non-halophiles and halophiles should be used, and, as ZoBell has shown with his hydrocarbon-attacking species, the inclusion of different carbon sources in the media may yield new varieties. The temperature of incubation is also of major importance, as shown by the existence of ZoBell's 80° strains and by experiments to be described.

### ISOLATION OF PURE CULTURES

It is comparatively simple to grow mixed cultures containing sulphate-reducing bacteria. It is not difficult to obtain almost pure cultures, but in general it is by no means easy to isolate absolutely pure strains. Full details of the various media and methods hitherto employed may be found in papers by Beijerinck (1895), van Delden (1908), Elion (1925), Gahl & Anderson (1928), Baars (1930), Starkey (1938) and Rittenberg (1941). Because some of these papers are not easily available and also because of the difficulties generally experienced by workers in this field, our methods are described in some detail; they include salient features of procedures adopted by other workers.

The general method used is based on that of Starkey (1938), which consists essentially in making enrichment cultures and then preparing deep agar ('shake') cultures or plates in various dilutions. A solid medium containing sulphate, lactate and a trace of ferrous salt is used; the sulphate-reducers form black colonies which can readily be picked off and placed in liquid medium. This apparently simple procedure rarely meets with immediate success except sometimes with thermophilic strains. It is difficult to obtain colonies free from contaminants, and it is frequently necessary to undertake a tedious succession of deep agar dilution cultures and platings out before pure cultures are obtained. We have found that the inclusion of 8%  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  in the media eliminates most of the contaminating organisms in mixed cultures and greatly facilitates subsequent purification of the sulphate-reducers.

### *Methods*

#### *Anaerobic cultivation*

Sulphate-reducing bacteria are said to be strict anaerobes, though Bunker (1939) states that 'they can be active in very restricted areas of oxygen deficiency in close proximity to regions of abundant oxygen supply'. Some of our own experiments indicate, not conclusively, that growth may occur in the presence of 2-8 p.p.m. of oxygen. We have also grown pure strains aerobically in shallow (1 cm.) layers of medium to which 0.025% ascorbic acid had been added (see Kligler & Guggenheim, 1938). Most soils incubated with medium A (below) reduce sulphate even if no anaerobic precautions are taken, presumably

because the aerobic organisms also present deoxygenate the medium. Nevertheless, growth is much better when oxygen is rigorously excluded, and the choice of an efficient and convenient anaerobic technique is most important.

The simplest technique for crude cultures is the use of stoppered bottles completely filled with medium freed from dissolved oxygen by boiling and rapidly cooled immediately before use. This method is unsuitable for work with pure cultures owing to the ease with which contamination takes place. With normal aseptic precautions the majority of bottle cultures show significant contamination when plated aerobically on nutrient agar. Even with elaborate precautions the incidence of contamination is still too great for general work. This objection does not apply to ordinary culture tubes plugged with cotton-wool soaked in alkaline pyrogallol and sealed with a rubber bung, but the method is cumbrous when large numbers of tubes are used. Of the various anaerobic techniques available, the McIntosh & Fildes anaerobic jar, with an atmosphere of hydrogen containing 5% carbon dioxide, was found to be the most satisfactory and efficient for general use. In order to prevent poisoning of the palladium catalyst by hydrogen sulphide, it is necessary to place a dried pad of absorbent cotton-wool impregnated with lead acetate between the cultures and the catalyst.

### *Crude cultures*

*Sources.* The sources investigated were: all kinds of soils; river, sea, pond and tap waters; water from gas-holders and oil-storage tanks; oil-well waters; sewage; marine sediments.

*Media.* For crude and pure cultures we used media based on the following:

*Medium A* (Baars, 1930):  $K_2HPO_4$ , 0.5 g.;  $NH_4Cl$ , 1 g.;  $CaSO_4$ , 1 g.;  $MgSO_4 \cdot 7H_2O$ , 2 g.; sodium lactate (70% solution), 5 g.;  $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ , 0.5 g.; tap water, 1 l.; pH 7.0–7.5, which should be confirmed after sterilization. This medium gives a considerable precipitate on sterilization for 20 min. at 20 lb./sq.in. which is no disadvantage in crude cultures, but may be with pure cultures when, for example, growth is determined by turbidity measurements.

*Medium B* (Starkey, 1938):  $K_2HPO_4$ , 0.5 g.;  $NH_4Cl$ , 1 g.;  $Na_2SO_4$ , 1 g.;  $CaCl_2 \cdot 2H_2O$ , 0.1 g.;  $MgSO_4 \cdot 7H_2O$ , 2 g.; sodium lactate (70% solution), 5 g.;  $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ , 0.5 g.; distilled water, 1 l. (Starkey used tap water); pH 7.0–7.5. This medium gives only a slight precipitate on autoclaving and may be filtered clear without significant loss and resterilized.

It is preferable not to incorporate the ferrous salt in the stock media, but to sterilize a 1% solution by steaming 1 hr. on 3 successive days, and to add 5 ml. supernatant liquid to each 100 ml. medium immediately before use. Media A and B are suitable for growing obligate and facultative non-halophilic strains in crude cultures. For halophilic strains 1–3% NaCl is added or tap or distilled water replaced by sea water. Other saline media are described by Rittenberg (1941) and ZoBell (1947).

*Addition of sulphite.* A varied microflora can be observed when soils are incubated anaerobically at 30° in media A and B, but comparatively few characteristic sulphate-reducing vibrios can be seen. Contaminations are very



persistent and cannot be eliminated by repeated transfers to fresh medium. Baars (1980) considerably diminished the number of contaminating organisms by adding 4 ml. of 0.1 N- $\text{H}_2\text{S}$  to 60 ml. medium, but Starkey (1988) did not find this method useful for isolation purposes.

We examined the effect of adding various concentrations of  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  to medium A on the microflora of crude cultures from soils incubated at 30°. The effect varied with different soils, but in general the development of sulphate-reducers was not appreciably inhibited by concentrations up to 5 %, while very considerable purification took place. With some soils almost pure cultures were immediately obtained, and subsequent isolation of pure strains was comparatively easy.

Clearly no obligate non-halophilic sulphate-reducer can develop in the sulphite medium, and if such a strain is desired the sulphite-free medium must be used. It is now our practice, when making crude cultures at 30° for the isolation of pure cultures, to incubate the inoculum in medium A with and without 3 %  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ . Most soils yield crude cultures in both media. When, as sometimes occurs, growth in the sulphite medium is inhibited or very slight, subculture from the normal into sulphite medium will usually give rapid sulphate reduction and a simpler microflora. Crude cultures at 55° have a much simpler microflora and the addition of sulphite is unnecessary.

Sulphite solutions are unstable and sulphite media should be made up shortly before use. A freshly made strong solution (20–30 %) is sterilized by passing through a bacteriological filter and is added in appropriate quantity to sterile medium. For crude cultures solid sulphite may be dissolved in sterile medium immediately before use. The addition of 3 % of sulphite raises the pH of medium A from 7.2 to *c.* 8.4. It is advisable to adjust to pH 7.2 by the addition of about 2 ml. N-HCl/100 ml. medium. Sulphite in concentrations of 2–5 % causes a remarkable lengthening of the bacterial cell in sulphate-reducers, varying considerably with the individual strain. This effect will be more fully described in a future communication.

*Procedure.* The procedure is determined by the source of the inoculum and the type of strain required. Strains from a saline environment are best isolated on medium A or B with the addition of 2–3 % NaCl, though many halophilic strains can reduce sulphate in its absence. Most soils and sewages readily yield flourishing crude cultures of sulphate-reducers when 1–2 g. are incubated anaerobically with media A and B, with and without 3 %  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ . For these cultures it is convenient to employ stoppered bottles containing 30–50 ml., but cultivation in ordinary culture test-tubes in anaerobic jars is equally effective. Waters, on the other hand, vary enormously in the volume necessary to yield positive cultures. With a heavily contaminated soil water we have obtained sulphate reduction from inocula of 0.00001 ml.; with other waters 50 or 100 ml. must be incubated with the medium. Hence, with waters obviously heavily infected, e.g. smelling of  $\text{H}_2\text{S}$  and showing vibrios under the microscope, only 1–2 ml. need be used, but normally our practice with waters is to incubate 1, 10, 50 and 100 ml. samples. Bunker (unpublished observation) grew a strain from 25 ml. tap water. For the larger quantities we use stoppered bottles

of appropriate size or flasks containing alkaline pyrogallol plugs. To prevent undue dilution by the larger inocula, stronger media are used.

When considerable blackening of the medium has occurred, which may take 2-8 days, and sometimes a month, the culture is examined for vibrios. If vibrios predominate, which occurs sometimes in sulphite but rarely in non-sulphite medium, the culture is ready for the isolation of pure strains. If not, transfers are made into fresh medium, preferably using portions of the black sludge, which usually contains a greater concentration of vibrios than the supernatant liquid. Subculturing is continued until microscopical examination reveals a flourishing culture of vibrios; usually three or four transfers are necessary in the absence of sulphite.

*Temperature of incubation.* The temperature of incubation need not necessarily be near the normal environmental temperature in nature: the first thermophilic sulphate-reducer was obtained from mud in a frozen ditch (Elion, 1925). Crude cultures may be obtained over a very wide range of temperature. Most soils give positive results at 30° and 55°. Twelve different types of soil from various parts of Greater London all yielded flourishing cultures of sulphate-reducers when incubated with medium A at both temperatures; similar results have been obtained with soils from many different parts of the world.

The development of crude cultures at temperatures between 30 and 55° has been examined. Portions (1-2 g.) of a Teddington garden soil and of a Syrian soil were incubated anaerobically with medium A at 30, 35, 40, 45, 50 and 55°. All cultures showed considerable sulphate reduction and developed flourishing growths of sulphate-reducers. Other soils similarly incubated gave negative results initially at one or two intermediate temperatures, but on prolonged incubation all gave positive results.

It is difficult to explain these results. No pure strain known to us is capable of developing at all these temperatures without gradual acclimatization. It is most unlikely that more than one or two different species are present in the same sample of soil. More probably there are different strains of the same species adapted to various restricted ranges of temperature, but we have no explanation of how the adaptation has occurred.

#### *Isolation of pure strains from crude cultures*

Starkey (1938) described the fortuitous isolation of pure cultures by incubating at 30° for 443 days without subcultivation; presumably all contaminating organisms had been destroyed by the prolonged action of hydrogen sulphide. Rittenberg (1941) did not obtain complete purification of crude cultures by storing for 29 months, though partial purification can undoubtedly be effected in this manner. We have used the method of preparing deep agar ('shake') and plate cultures of various dilutions of the crude culture in 0.85 % saline, using medium B with 2 % agar, filtered clear. With 3 %  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  the medium gives more rapid isolation, but in the few instances where growth is inhibited, the medium without sulphite is used.

Nine ml. of cooled but still liquid agar medium are mixed with 1 ml. 30 %

sulphite (sterilized by filtration), 0.2 ml. N-HCl (to bring to *c.* pH 7) and then 1 ml. culture dilution, in that order. It is unnecessary to take special anaerobic precautions with the deep agar tubes, as sulphate-reducers grow in all but the upper  $\frac{1}{4}$  in. of the medium. Plates are incubated in McIntosh & Fildes jars; special care should be taken to exhaust the jars thoroughly before admitting the gas mixture. The cultures are incubated at the required temperature until black colonies appear. Both tubes and plates are seeded because growth on solid media is haphazard, and discrete colonies are better obtained sometimes in tubes, sometimes on plates. Colonies which under low power of the microscope appear to be free from neighbouring contaminating colonies are transferred to tubed liquid medium, with and without sulphite, and incubated anaerobically. Stoppered bottles should not be used, for the reasons already given. Starkey (1938) found that the colonies frequently failed to grow when transferred direct into liquid. We have had comparatively few failures by using either medium A or B containing 0.1 % Difco yeast extract, which greatly stimulates growth.

Cultures so obtained are rarely pure except in media containing sulphite, and then not always. When contaminations are found, further dilutions must be made either from the liquid culture or from another colony and fresh deep agar and plate cultures made. The process is repeated until the culture is pure. Owing to the heteromorphic character of sulphate-reducers, microscopical examination is not infallible and confirmation of absence of contamination should be made by plating on various media (nutrient agar, glucose agar, etc.) incubated aerobically and anaerobically. The isolation of pure thermophilic strains is usually rapidly accomplished without the addition of sulphite to the medium. H. J. Bunker & A. C. Thaysen (unpublished observation) isolated a pure strain by means of a micromanipulator. This technique is undoubtedly superior to any other but the apparatus is not generally available.

### *Stock cultures*

Pure cultures of sulphate-reducing bacteria may be kept alive on artificial media for long periods. Liquid mesophilic cultures in medium A stored at room temperature for a year grew vigorously on subculture. Slope cultures on solid medium (medium A + 0.1 % Difco yeast extract + 2 % agar) in stoppered tubes containing alkaline pyrogallol plugs grew well after 18 months' storage. Thermophilic spore-forming strains, kept at 55°, were viable after 18 months in liquid medium (medium A) and after 2 years in agar stabs (medium A + 2 % agar). Starkey (1938) quotes other examples of longevity. There is, therefore, no difficulty in keeping stock cultures on artificial media both liquid and solid. For preserving the original characteristics of different strains, however, prolonged cultivation on such media is open to grave objection, and sterile clay and sand were tested as media for stock cultures.

Tubes containing 5 g. each, some of moist clay, others of sand, were sterilized by autoclaving for 2 hr. at 15 lb./sq.in. Stock cultures of four pure strains were prepared by adding 5 drops of actively growing cultures. The tubes were then

plugged with cotton-wool saturated with alkaline pyrogallol, sealed with a rubber bung and stored at room temperature. After 4 months all four strains on clay grew vigorously when transferred to medium A; no growth was obtained with the sand cultures. After 9 months all four clay cultures were still viable, but three failed to develop after 12 months, and the fourth after 18 months. It is advisable, therefore, to make fresh clay cultures after *c.* 9 months.

#### HETEROTROPHIC GROWTH

*Liquid media.* For crude cultures and isolation of pure strains the medium is deliberately kept simple and free from stimulants which will also favour the development of other organisms. Although there is little difficulty in obtaining growth of pure cultures in media A and B, the amount of cell material is relatively slight, despite the impression of good growth given by precipitation of ferrous sulphide. The latter, which appears as a bulky black precipitate in the cultures, though convenient for demonstrating sulphate reduction, is inconvenient in biochemical and other investigations. Early in our work, we were confronted with the problem of obtaining large quantities of cells free from sulphide and other contaminating material. For this purpose we needed a clear medium giving vigorous growth but containing ferrous iron below the saturation concentration for ferrous sulphide. Bunker (1939) found that yeast, yeast water and filtered yeast culture stimulated growth. This suggested the addition of yeast extract. Eventually, the following medium was found to give consistently rapid and abundant growth, considerable turbidity being obtained within 48 hr. with both mesophilic and thermophilic cultures.

*Medium C.*  $\text{K}_2\text{HPO}_4$ , 0.5 g.;  $\text{NH}_4\text{Cl}$ , 1 g.;  $\text{Na}_2\text{SO}_4$ , 1 g.;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g.; sodium lactate (70 % solution), 3.5 g.; Difco yeast extract, 1 g.;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 g.; distilled water, 1 l.; bring to pH 7.5. Autoclave for 20 min. at 20 lb./sq.in. This gives a slight precipitate, but a stable clear solution is obtained by filtering and resterilizing.

*Iron requirements.* The following experiments show that iron is essential for growth, but only in small quantity.

Medium C, made up with no added ferrous salt, was rendered 'biologically free' of iron by the method of Waring & Werkman (1943), in which iron complexes formed by the addition of 8-hydroxyquinoline are removed by chloroform extraction. The iron-free medium was placed in test-tubes which had been cleaned with chromic + sulphuric acid, well washed in distilled water and finally rinsed in conductivity water. A culture in medium C, containing no added ferrous salt, was incubated for 8 days at 30°, centrifuged, the cells washed with the same medium, and inoculated into the iron-free medium. No growth was obtained, though normal growth occurred on addition of 0.0002 %  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ .

Good growth was also obtained in six successive subcultures into medium C made up without added ferrous salt and using A.R. grade materials (except the  $\text{K}_2\text{HPO}_4$  and sodium lactate) and conductivity water in carefully cleaned glassware. Traces of iron present as impurities in the materials used were thus

sufficient for normal growth. Although no quantitative determinations of the iron contents of the individual constituents were made, qualitative experiments with 8-hydroxyquinoline showed that sodium lactate was the chief source of iron.

*Solid media.* Growth on the surface of solid media is neither so successful nor so rapid and abundant as in liquid media. It is somewhat capricious; very occasionally luxuriant growth was obtained, and there were fairly frequent unexplained failures. The appearance of abundant growth is often given by intense blackening of media to which ferrous iron has been added, but the quantity of cell material is frequently scanty. The best results were obtained by using a massive inoculum from a vigorous young liquid culture. Media A and B with 2% agar and 1% Difco yeast extract usually gave satisfactory growth, but the peptone-dextrose agar (peptone, 5 g.; dextrose, 10 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.5 g.;  $\text{Na}_2\text{SO}_4$ , 1.5 g.;  $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ , 0.1 g.; agar, 20 g.; tap water, 1 l.) of Starkey (1938) gave consistently better results. Attempts were made to grow large quantities of cells free from sulphide on the various solid media containing no added ferrous salt, but were not successful.

#### AUTOTROPHIC GROWTH

Pont (1939) claimed to have grown sulphate-reducers in a mineral medium containing steel wool. It is possible, as Hadley (1943) suggests, that organic material was present as vegetable oil on the steel or may have been introduced with the inoculum, and though apparently no carbon source was added, there was probably dissolved carbon dioxide in the medium. Hadley was unable to confirm Pont's results. Czurda (1940) also claimed to have grown sulphate-reducers in a mineral medium in the absence of hydrogen; his cultures were admittedly impure, and ZoBell (1946*b*) suggested that the organisms were using the paraffin wax employed to exclude oxygen from the cultures. van Niel (1943) wrote that 'decisive experiments to show development of the sulphate-reducing bacteria in mineral media, on the basis of oxidation of hydrogen with sulphate, have not yet been carried out'.

Starkey & Wight (1945) were the first to produce substantial evidence that sulphate-reducers are facultative autotrophs. They were able to show that enriched and partly purified cultures grew in an inorganic medium in the presence of hydrogen; the sole carbon source was bicarbonate, and sulphate was reduced to sulphide. They suggested that the necessary energy was obtained by the oxidation of hydrogen. Starkey & Wight stated, however, that 'the results are not entirely satisfactory in that it has not been shown conclusively that pure cultures of the sulphate-reducing bacteria can bring about these reactions'. It had previously been shown by Stephenson & Stickland (1931) that resting non-proliferating cells of a strain of sulphate-reducers isolated by them contained a hydrogenase, but there was no indication that growth was possible by virtue of this reaction.

As briefly reported (Butlin & Adams, 1947) we grew one thermophilic and four mesophilic strains through many subcultures in a strictly mineral

medium in an atmosphere of hydrogen containing 5 % carbon dioxide; no growth took place when hydrogen was excluded. Growth also occurred when sulphate in the medium was replaced by sulphite, thiosulphate and elementary sulphur. Furthermore, the gaseous hydrogen need not be provided as such; it could be produced in the medium by immersing metallic iron in the mineral salt solution. Our experiments with pure cultures show that growth and sulphate reduction occur when rods of mild steel are placed in the inoculated mineral salt medium and incubated anaerobically with no added hydrogen. The cultures remained pure throughout the experiments. Very stringent precautions were taken to exclude all traces of organic material.

### *Experimental*

*Medium D.*  $\text{K}_2\text{HPO}_4$ , 1 g.;  $\text{NH}_4\text{Cl}$  (A.R.), 1 g.;  $\text{CaSO}_4$ , 1 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (A.R.), 2 g.;  $\text{NaHCO}_3$  (A.R.), 1 g.; conductivity water, 1 l. The  $\text{NaHCO}_3$  is added separately after sterilization by filtration of 10 % solution (10 ml. to 1 l.). pH 7.0–7.5. 1 % solution  $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$  (A.R.) sterilized by 1 hr. steaming on 3 successive days; 5 ml. supernatant liquid added to each 100 ml. medium immediately before use.

*Glassware.* All glassware used in preparation of media and for culture tubes was cleaned in chromic acid, washed in distilled water and finally rinsed in conductivity water. Cotton-wool plugs normally used in culture tubes and flasks containing medium were replaced by loosely-fitting beakers or tubes cleaned in chromic acid, etc. Pipettes were plugged with acid-washed glass wool, placed inside large glass tubes and autoclaved.

*Incubation* was in McIntosh & Fildes anaerobic jars containing atmosphere of hydrogen with 5 %  $\text{CO}_2$ ; mesophilic strains incubated at 30°, thermophilic at 55°. Cultures of five strains, one thermophilic and four mesophilic, growing in medium A were first cultivated in medium D (made with distilled water and contained in tubes plugged with cotton-wool) without taking the elaborate precautions described above. A period of about 8 days elapsed before blackening occurred, showing reduction of sulphate to sulphide. The cultures were taken through several subcultivations. The rate of growth increased considerably, and by the third or fourth subculture reasonably good growth was obtained in 2–3 days. After six subcultivations, when there could have been only very slight traces of organic material present, a change was made to the all-glass culture tubes and to the special precautions for preparing media, etc., described above. Growth persisted through at least six subcultures and showed no signs of failing. Throughout the experimental period, the purity of the cultures was proved microscopically and by seeding on nutrient agar and glucose broth and in broth and glucose broth, aerobically and anaerobically. No growth could be obtained from similar cultures incubated in anaerobic jars containing no hydrogen and from which oxygen was removed by alkaline pyrogallol.

All five strains (four mesophilic, one thermophilic) must therefore be regarded as facultative autotrophs. It should be emphasized that growth is not nearly

so abundant as in heterotrophic conditions, and for this reason it has not yet been found possible to demonstrate the presence of a hydrogenase in washed suspensions of cells grown autotrophically. However, experiments in Warburg manometers with washed suspensions of the mesophilic strains grown heterotrophically show that hydrogen is absorbed in the presence of sulphate, with formation of sulphide. The presence of a hydrogenase in the autotrophic strains may reasonably be presumed.

*Sulphite, thiosulphate and elementary sulphur as hydrogen acceptors*

In heterotrophic growth of sulphate-reducers, sulphite, thiosulphate and elementary sulphur can be substituted for sulphate in the medium, reduction to sulphide taking place in each case (Baars, 1930). Experiments were made to determine whether similar substitutions could be made in media supporting strictly autotrophic growth.

The following basal medium containing no sulphate was made up:  $K_2HPO_4$ , 1 g.;  $NH_4Cl$  (A.R.), 1 g.;  $CaCl_2 \cdot 6H_2O$  (A.R.), 0.1 g.;  $MgCl_2 \cdot 6H_2O$  (A.R.), 1.1 g.;  $NaHCO_3$  (A.R.), 1 g., added separately after sterilization of 10% solution by filtration; conductivity water, 1 l. pH adjusted to 7.0–7.5 after addition of bicarbonate.  $FeCl_2$  was used instead of  $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$  as indicator. A 2%  $FeCl_2$  solution was boiled for 5 min. and 1 drop added to each 10 ml. medium before use.

Sulphite medium was prepared by adding 20 ml. of 20%  $Na_2SO_3 \cdot 7H_2O$  (sterilized by filtration) to each litre of basal medium. Owing to the instability of sulphite solutions, filtration and addition to medium was performed rapidly and immediately before inoculation. Thiosulphate medium was prepared by adding 4 g.  $Na_2S_2O_3 \cdot 5H_2O$  (A.R.)/l. to the basal medium; sulphur medium by adding c. 0.1 g. precipitated sulphur to each 10 ml. basal medium in culture tubes and sterilizing by steaming on 8 successive days before addition of bicarbonate. The cultures were incubated in an atmosphere of  $H_2$  containing 5%  $CO_2$ .

The technique used was similar to that already described for autotrophic growth with sulphate as hydrogen acceptor, preliminary experiments being performed in culture tubes plugged with cotton-wool and the final subcultures carried out in all-glass culture tubes, with stringent precautions to eliminate extraneous organic material. Reasonably good growth with reduction to sulphide was obtained with four mesophilic strains at 30° and one thermophilic strain at 55°, except in the medium containing elementary sulphur, which showed definite blackening but very scanty growth. Uninoculated controls remained unchanged.

*Autotrophic growth in presence of metallic iron*

Starkey & Wight (1945) grew sulphate-reducing bacteria at 28° in mineral medium to which steel-wool had been added. Hydrogen is evolved through reaction with water when iron is immersed in the culture medium. As with the cultures growing autotrophically in an atmosphere of  $H_2$ , it was assumed that

the energy for growth was obtained by the oxidation of hydrogen and that sulphate acted as hydrogen acceptor. Starkey & Wight stated that their technique was not well adapted to pure culture work, and contaminants were present in all cultures at the end of the incubation period. In our experiments using metallic iron as the source of hydrogen, we used the technique already described for autotrophic growth, and our cultures remained pure.

Steel rods about 1 in. long were first cleaned by three immersions in pure benzene, then washed with hot distilled water, rinsed in conductivity water and finally sterilized by autoclaving in medium D before addition of bicarbonate. After inoculation with two drops of culture grown through several subcultures in mineral medium contained in all-glass culture tubes, the cultures were incubated in an anaerobic jar containing alkaline pyrogallol. Four mesophilic cultures were placed at 30° and one thermophilic culture at 55°. After 2-3 days a black precipitate began to appear on the surface of the steel rod and growth, though scanty, was definite and persisted through several subcultures. Uninoculated controls remained unchanged.

#### DISCUSSION

No attempt has been made to elucidate the autotrophic metabolism of the strains investigated, but in view of the fact that hydrogen is essential and that the corresponding heterotrophic mesophilic strains contain a hydrogenase, it may reasonably be inferred that the oxidation of hydrogen by the hydrogenase system is the source of energy. The energy released must be used to fix CO<sub>2</sub> for growth purposes and also to supply the energy for the reduction of sulphate (or sulphite, thiosulphate and sulphur) to sulphide, a reaction which appears to be essential to the life of the cell.

Some problems of considerable biochemical interest are raised, in particular the mechanism which must link the energy-releasing and energy-absorbing systems. The reduction of sulphate to sulphide by stages suggested by Kluyver (1931), but not yet experimentally established, is presumably linked to the oxidation of hydrogen by hydrogenase, but there remains the problem of synthesis of cell materials from bicarbonate as sole carbon source. LePage & Umbreit (1943) showed that when the aerobic obligate autotroph *Thiobacillus thiooxidans* oxidizes sulphur to sulphuric acid, an energy-rich adenosine-3-triphosphate is formed, and that energy obtained from the energy-rich phosphate bonds may be used for growth purposes. The possibility that a similar mechanism operates in the autotrophic metabolism of sulphate-reducers might be explored.

Another matter for speculation is the manner whereby elementary sulphur, which is insoluble in the growth medium, is reduced to sulphide during heterotrophic or autotrophic growth of sulphate-reducers in apparent violation of the principle that energy transformations take place at the surface of, or within the cell. It has been suggested that cells of *Th. thiooxidans* contain unsaturated-fat globules which dissolve sulphur by direct contact and so bring it into the



cell for oxidation (Umbreit, Vogel & Vogler, 1942), though this speculation has been partially disputed by Knaysi (1943).

The utilization of the hydrogen evolved when metallic iron is immersed in mineral medium provides additional evidence for the presumed role of sulphate-reducers in anaerobic corrosion of ferrous pipes, namely, that of depolarizing the cathodic elements of electro-chemical systems on the surface of the metal. It by no means follows that the organisms taking part in the corrosion process are living autotrophically, for corrosion of this type is usually more severe in the presence of the products of cellulose decomposition, which provide oxidizable food material for the organisms. It is probable, therefore, that future investigations will show that a hydrogenase plays an important part in the heterotrophic as well as the autotrophic metabolism of sulphate-reducers. It is improbable that all sulphate-reducers are capable of autotrophic growth, for Stephenson (1947) states that a hydrogenase is not invariably found in this group. The possession or non-possession of a hydrogenase might serve as a basis for differentiation into separate species.

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## Some Biological Properties of Trichothecin, an Antifungal Substance from *Trichothecium roseum* Link

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**SUMMARY:** The production of trichothecin by strains of *Trichothecium roseum* was greatly increased when nutrients such as yeast extract, malt extract or corn-steep liquor were added to Czapek-Dox medium containing 5 % (w/v) glucose. Ammonium salts, especially ammonium tartrate, were better than nitrate as source of nitrogen, and high trichothecin titres were also obtained with asparagine or glycine as source of nitrogen. Maximum yields of the order of 100 mg./l. were obtained on a modified Czapek-Dox medium containing ammonium tartrate instead of sodium nitrate and including 5 % (w/v) glucose and 1 % (v/v) of corn-steep liquor, in about 30 days, with 4 cm. depth of medium.

Trichothecin had no antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* or *Bacterium coli*. It was active against each of the twenty-seven species of fungi examined. The growth of *Penicillium digitatum*, the most sensitive species investigated, was completely inhibited at 0.64 mg./l., and 50 % germination of its spores took place at trichothecin concentrations between 0.30 and 0.75 mg./l., according to the age of the spores and other factors.

Antagonism between *Trichothecium roseum* Link and certain plant-pathogenic fungi was reported by Whetzel (1909), Boning (1933), Koch (1934) and Greaney & Machacek (1935). Koch (1934) stated that *T. roseum* actively parasitized the stroma of *Dibotryon morbosum* in the black-knot disease of various hosts. The influence of *Trichothecium roseum* on the pathogenicity of *Helminthosporium sativum* in experiments with cereals has been observed by Greaney & Machacek (1935). Antifungal activity of culture filtrates of *Trichothecium roseum* against *Botrytis allii* was reported by Brian & Hemming (1947). The substance responsible for this antagonism was isolated from culture filtrates of the fungus and its chemical properties described (Freeman & Morrison, 1948), and was given the name 'trichothecin'. The work described here is concerned with the most favourable conditions for the production of trichothecin, and with its biological properties. We first observed that a ten-fold dilution of the culture filtrate of a strain of *Trichothecium roseum* on beer-wort completely suppressed the germination of *Penicillium digitatum* spores. In cylinder-plates the growth of *Aspergillus niger* mut. *schiemani*, *Paecilomyces varioti*, *Fusarium graminearum* and *Penicillium hagemi* was inhibited, though less markedly than that of *P. digitatum*. Antagonism of *Trichothecium roseum* towards *Penicillium* spp., both on liquid and solid media, was frequently observed, the mycelia of the latter being ultimately completely overgrown by *Trichothecium roseum*.

### EXPERIMENTAL

**Strains of *Trichothecium roseum*.** Four strains of *T. roseum* were used; they were isolated in this laboratory from specimens of cut wood collected locally. When freshly isolated on beer-wort agar medium all showed the characteristic

macroscopic appearance of *T. roseum*. The colonies, which were initially white and thinly floccose, rapidly developed a rose pink conidial zone. The erect conidiophores carried terminal clusters of two-celled oval conidia about  $20 \times 10 \mu$ , each with a nipple-like projection at the point of attachment. Microscopically the various strains were indistinguishable; their various colonial appearances were as follows.

*Strain no. F109.* Colonies on beer-wort agar were thinly floccose, white at first, rapidly becoming pink and velvety as conidia developed. On liquid Czapek-Dox medium (5 % (w/v) glucose) supplemented by 1 % (v/v) corn-steep liquor (CSL), initially produced thick smooth convoluted felt, deep pink in colour. After a number of subcultures on wort agar, the mycelium on liquid medium became largely sterile, compact, white and convoluted with a few small pink conidial areas. This change coincided with a decrease from 30 to 5 mg./l. in the trichothecin production on the above medium. The original

Table 1. *Maximum trichothecin production and other data of strains of Trichothecium roseum*

| Strain no. | Source                                      | Date isolated | Maximum trichothecin production under conditions described in Table 7 on medium 4 cm. deep (mg./l.) |
|------------|---|---------------|---|
| F109       | Sycamore ( <i>Acer pseudoplatanus</i> ) log | June 1945     | 25  |
| F198       | Unidentified wooden log                     | August 1946   | 150   |
| F227       | Dead stem of <i>Rosa canina</i>             | December 1946 | 110   |
| F271       | Sycamore ( <i>Acer pseudoplatanus</i> ) log | January 1948  | 20  |

activity was restored when sodium nitrate was replaced by ammonium tartrate as nitrogen source, but the mycelium remained largely sterile, although conidia developed normally on wort agar.

*Strain no. F198.* Colonies on wort agar were thin, pale pink with numerous areas of sterile floccose mycelium. After 3-4 subcultures on wort agar only white floccose sterile mycelium was produced. No antifungal activity was detected when this strain was grown on Czapek-Dox glucose CSL medium, but high concentrations of trichothecin were obtained with the ammonium tartrate-containing medium.

*Strain no. F227.* Colonies on wort agar were similar to those of strain F109, but with occasional floccose sterile areas. On liquid glucose CSL Czapek-Dox medium the mycelium was mainly pink with heavy development of conidia, and the surface was much rougher than that of strain F109. There was occasional development of loose, white floccose areas with delayed conidial production. On the ammonium tartrate-containing medium similar but heavier growth took place.

*Strain no. F271.* Colonies on wort agar and conidial production were similar to strain F109. On the ammonium tartrate-containing medium the mycelium resembled that of strain F109 in texture, but conidial development took place evenly over the whole surface.

*Assay of trichothecin*

(a) *By inhibition of germination of Penicillium digitatum conidia.* The basis of the method was described by the Committee on Standardization of Fungicidal Tests of the American Phytopathological Society (1947), and its application to conidia of *Botrytis allii* by Brian & Hemming (1945). *Penicillium digitatum* conidia were used as test material and Czapek-Dox medium containing 5 % (w/v) glucose at pH 4.2 was employed as germination medium. In preliminary tests, considerable variations were observed in the sensitivity towards the antifungal compound of different batches of spore suspensions (Table 2). This variation

Table 2. *Variation in sensitivity to trichothecin of Penicillium digitatum spore suspensions*

| No. of culture | Age of culture (days) | Percentage germination of spores in presence of trichothecin (mg./l.) |      |      |      |      | ED 50 (mg./l.) |
|----------------|-----------------------|---|------|------|------|------|----------------|
|                |                       | 1.25  | 0.63 | 0.31 | 0.16 | 0.08 |                |
| 1              | 7                     | 10  | 60   | 100  | —    | —    | 0.75           |
| 2              |                       | 1   | 10   | 50   | 80   | 100  | 0.29           |
| 3              |                       | 5   | 50   | 100  | —    | —    | 0.63           |
| 4              |                       | 10  | 60   | 100  | —    | —    | 0.75           |
| 5              |                       | 5   | 30   | 70   | 100  | —    | 0.44           |
| 6              |                       | 5   | 20   | 50   | 80   | 100  | 0.31           |
| 7              | 14                    | 1   | 40   | 80   | 100  | —    | 0.53           |
| 8              |                       | 0   | 20   | 50   | 100  | —    | 0.38           |
| 9              |                       | 5   | 50   | 100  | —    | —    | 0.63           |

was overcome by use of a parallel series of standard concentrations of trichothecin with each set of assays, from which the concentration of the substance which permitted 50 % germination (ED 50) was determined for each batch of spores. The ED 50 was determined graphically by plotting the probit corresponding to the percentage germination against the logarithm of the concentration (Finney, 1947) and reading the ED 50 on the linear response curve thus obtained. The ED 50 is cited in the tables in mg./l.

(b) *By a cylinder-plate method.* The method was an adaptation of the cylinder-plate diffusion technique (Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey, 1941), in which a porcelain cylinder was embedded in beer-wort agar medium, sown with *P. digitatum* spores, in a Petri dish. The cylinder was filled with the test solution and the plate incubated for 3 days at 25°. The dose-response curve for trichothecin (Fig. 1), for concentrations in the range of 1.56–200 p.p.m., shows that there is a linear relationship between diameter of zone of inhibition and the logarithm of trichothecin concentration.

The method compares unfavourably with the spore-germination method. It is less precise because of the diffuse nature of the boundaries of the zones of inhibition, and because the zones are too large to allow both standard and test solutions to be set up on the same 9 cm. plate. It is also slow, since 3 days must elapse before results are obtained.

The data summarized in Tables 3 and 4 were obtained by this method.

*Factors which influence trichothecin production in liquid media*

The influence of a number of factors on trichothecin production in liquid media was investigated. The basal medium was Czapek-Dox containing 5% (w/v) glucose, sterilized by autoclaving in 350 ml. quantities (giving 17 mm. depth of medium) in 'Glaxo' bottles. The initial pH was 5.0. The cultures were

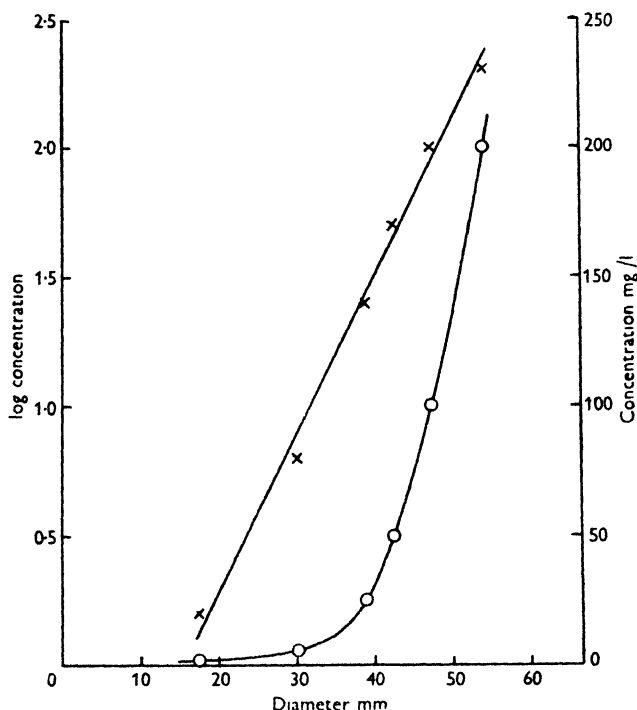


Fig. 1. Relationship between trichothecin concentration and diameter of zone of inhibition of *Penicillium digitatum* in cylinder-plate assay method. ○—○ zone diameter plotted against trichothecin concentration (mg./l.). ×—× zone diameter plotted against log concentration. Each diameter represents the mean reading from four plates.

inoculated with a suspension of *Trichothecium roseum* spores from about 7-day beer-wort agar slopes. The cultures were incubated at 25° in the dark, and samples of the filtrate assayed at intervals. In the absence of added organic nutrients growth was slow and sparse. To produce rapid growth and heavy development of mycelium nutrients such as yeast extract, malt extract, beer wort and CSL were necessary. In preliminary experiments CSL appeared to be most effective in stimulating development of trichothecin.

**Corn-steep liquor concentration.** The effect of CSL concentrations in the range 0.1–2% (v/v) at initial pH values of 4.0, 6.1 and 7.6 was determined (Table 3). In the absence of CSL only traces of activity were produced. Addition of 0.1% (v/v) resulted in a striking increase of activity, but further increase of CSL above 0.2% (v/v) produced little further increase in activity. Within the

range pH 4.0-7.6, initial pH had no significant effect on trichothecin production. In the subsequent experiments the various media contained 1 % (v/v) of CSL.

Table 3. *Influence of corn-steep liquor concentration on production of trichothecin by Trichothecium roseum strain F109 in 'Glaxo' bottle cultures*

Trichothecin was assayed by the cylinder-plate method.

| pH  | CSL<br>(%, v/v) | Period of incubation (days)            |      |      |       |
|-----|-----------------|--|------|------|-------|
|     |                 | Concentration of trichothecin (mg./l.) |      |      |       |
|     |                 | 6                                      | 8    | 12   |       |
| 4.0 | 0               | —                                      | —    | —    | Trace |
|     | 0.1             | —                                      | 4.2  | 20.0 | 34.0  |
|     | 0.2             | —                                      | 6.8  | 17.0 | 50.0  |
|     | 0.5             | 1.2                                    | 6.8  | 14.0 | 40.0  |
|     | 1.0             | Trace                                  | 7.8  | 12.0 | 34.0  |
|     | 2.0             | Trace                                  | 10.0 | 12.0 | 40.0  |
| 6.1 | 0               | —                                      | —    | 1.4  | 4.2   |
|     | 0.1             | —                                      | 1.8  | 3.5  | 12.0  |
|     | 0.2             | 1.2                                    | 6.0  | 7.8  | 41.0  |
|     | 0.5             | 2.0                                    | 6.0  | 16.0 | 41.0  |
|     | 1.0             | —                                      | 6.0  | 14.0 | 60.0  |
|     | 2.0             | Trace                                  | 10.0 | 14.0 | 50.0  |
| 7.6 | 0               | —                                      | —    | —    | —     |
|     | 0.1             | 2.0                                    | 6.0  | 6.0  | 20.0  |
|     | 0.2             | 3.8                                    | 10.0 | 7.8  | 40.0  |
|     | 0.5             | Trace                                  | 3.5  | 8.8  | 30.0  |
|     | 1.0             | Trace                                  | 2.7  | 6.0  | 34.0  |
|     | 2.0             | Trace                                  | 4.7  | 11.0 | 60.0  |

*Carbon source.* Comparison of glucose, sucrose, lactose and glucose + lactose as carbon sources (Table 4) showed that maximum trichothecin production took place with sucrose and that lactose was unsatisfactory.

Table 4. *Effect of source of carbon on trichothecin production by Trichothecium roseum strain F109 in basal medium enriched by various sugars*

Trichothecin was assayed by the cylinder-plate method.

| Carbon source and<br>% (w/v) conc. | Period of incubation (days)         |       |       |     |
|------------------------------------|-------------------------------------|-------|-------|-----|
|                                    | Trichothecin concentration (mg./l.) |       |       |     |
|                                    | 6                                   | 10    | 18    | 16  |
| Glucose, 5                         | 3.4                                 | 2.4   | 5.2   | 3.1 |
| Sucrose, 5                         | 12.0                                | 9.0   | 16.0  | 4.7 |
| Lactose, 5                         | < 1.0                               | < 1.0 | < 1.0 | —   |
| Glucose, 2.5; lactose, 2.5         | 2.0                                 | 2.2   | 3.1   | —   |

— Not tested.

*Nitrogen source.* The nature of the nitrogen source in the medium had considerable influence on the production of trichothecin. *T. roseum* strain F227 was grown on a series of media based on the Czapek-Dox formula, containing 5 % (w/v) of glucose and 1 % (v/v) of CSL, in which the normal nitrogen source,

$\text{NaNO}_3$ , was replaced by the equivalent amounts of  $\text{KNO}_3$ ,  $\text{NH}_4\text{NO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , ammonium tartrate, asparagine, urea and glycine (Table 5). The ammonium ion was superior to nitrate as a source of nitrogen, except when supplied in the form of ammonium sulphate, in which case the pH rapidly fell to about pH 2, presumably because of the liberation of sulphuric acid. Ammonium tartrate was the best nitrogen source for trichothecin production and was used in the preparation of trichothecin on a relatively large scale. Asparagine and glycine media both produced high titres of trichothecin, but the maxima were reached more slowly than with ammonium tartrate. Only a trace of activity developed when the medium contained urea as source of nitrogen. In a separate experiment, a medium containing the equivalent concentration of acetamide gave trichothecin production of the same order as that obtained with nitrate nitrogen.

Table 5. *Effect of nitrogen source on production of trichothecin in cultures of Trichothecium roseum strain F227*

Sodium nitrate in the Czapek-Dox formula was replaced by equivalent concentrations of other nitrogen sources in media containing glucose (5 %, w/v) and CSL (1 %, v/v). Trichothecin was assayed by the spore-germination method. Each result represents the mean of three cultures.

| Nitrogen source   | Conc. (g./l.) | Period of incubation (days)         |      |      |      |     |     |     |     |
|-------------------|---------------|-------------------------------------|------|------|------|-----|-----|-----|-----|
|                   |               | 4                                   | 7    | 10   | 13   | 4   | 7   | 10  | 13  |
|                   |               | Trichothecin concentration (mg./l.) |      |      |      | pH  |     |     |     |
| Potassium nitrate | 2.5           | —                                   | —    | 2.4  | 3.3  | 6.2 | 6.1 | 6.3 | 6.5 |
| Ammonium nitrate  | 0.6           | 2.6                                 | 25.0 | 56.0 | 50.0 | 4.3 | 5.9 | 6.5 | 6.9 |
| Ammonium sulphate | 1.5           | 2.5                                 | 8.0  | 5.1  | 5.0  | 3.5 | 2.4 | 2.0 | 2.1 |
| Ammonium tartrate | 2.2           | —                                   | 25.0 | 34.0 | 77.0 | 6.0 | 5.5 | 5.5 | 6.5 |
| Asparagine        | 1.4           | —                                   | —    | 6.8  | 35.0 | 6.5 | 6.4 | 6.3 | 6.9 |
| Urea              | 0.7           | —                                   | —    | 0.7  | —    | 7.0 | 7.5 | 7.3 | 8.0 |
| Glycine           | 1.8           | —                                   | —    | 18.0 | 55.0 | 6.0 | 6.6 | 6.5 | 6.8 |

— Not tested.

**Glucose concentration.** The effect of initial concentrations of glucose in the range 1–20 % (w/v) was determined in a medium containing 1 % (v/v) CSL and Czapek-Dox salts in which sodium nitrate was replaced by the equivalent quantity of ammonium tartrate (Table 6). With low concentrations (1 or 2 % (w/v)) of glucose, trichothecin concentrations rose to a maximum after 6 and 10 days, respectively, and thereafter fell equally rapidly to zero. The highest concentration was obtained with 5 % (w/v) glucose and was only slightly less with 10 % (w/v) initial glucose. With 20 % (w/v) initial glucose, both the rate of trichothecin production and the maximum concentration were much lower than with 5 and 10 % (w/v) initial sugar concentrations.

**Depth of medium.** The maximum practicable depth of medium for trichothecin production and isolation was determined in the medium used in the previous experiment, containing 5 % (w/v) initial glucose. The results (Table 7) show that although trichothecin concentration attained a maximum more slowly in the deeper media it was at least as high as in the shallower media.



*Biological properties of trichothecin*

Trichothecin had no antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* or *Bacterium coli*, at a concentration of 400 mg./l. (saturated aqueous solution) and pH 7.0, as determined by the cylinder-plate method (Abraham *et al.* 1941). Its antifungal activity is exhibited against Fungi

Table 6. *Effect of initial glucose concentration on trichothecin production by Trichothecium roseum strain F 227*

The fungus was grown on the following medium: ammonium tartrate, 2.2 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g.;  $\text{K}_2\text{HPO}_4$ , 1.0 g.;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g.; KCl, 0.5 g.; corn-steep liquor, 10 ml.; glucose, 10–200 g.; water to 1000 ml.; pH 5.0. Trichothecin was assayed by the spore-germination method.

| Glucose<br>initial<br>concentration<br>% (w/v) | Trichothecin concentration<br>(mg./l.) after (days) |      |    |      |    |    | pH after (days) |     |     |     |     |     |
|--|---|------|----|------|----|----|-----------------|-----|-----|-----|-----|-----|
|  | 4   | 7    | 10 | 13   | 17 | 24 | 4               | 7   | 10  | 13  | 17  | 24  |
| 1  | 19.0  | 20.0 | 11 | 2.5  | —  | —  | 5.0             | 6.5 | 7.1 | 7.7 | —   | —   |
| 2  | 24.0  | 30.0 | 39 | 25.0 | 13 | 3  | 5.4             | 6.6 | 7.3 | 7.7 | 8.5 | 9.1 |
| 5  | 24.0  | 45.0 | 33 | 34.0 | 61 | 29 | 5.1             | 6.7 | 7.2 | 7.3 | 8.3 | 8.6 |
| 10   | 27.0  | 41.0 | 45 | 50.0 | 38 | 33 | 4.8             | 6.5 | 7.0 | 7.3 | 8.4 | 8.2 |
| 20   | 1.3   | 4.7  | 13 | 26.0 | 33 | 35 | 4.1             | 6.0 | 6.7 | 7.0 | 8.2 | 8.1 |

— Not tested.

Table 7. *Effect of depth of medium on trichothecin production by Trichothecium roseum strain F 227*

Medium as in Table 6, with 5% (w/v) glucose. Trichothecin was assayed by the spore-germination method.

| Depth of<br>medium<br>(cm.) | Volume of<br>medium per<br>bottle<br>(ml.) | Period of incubation (days)         |      |    |    |    |     |    |
|-----------------------------|--|-------------------------------------|------|----|----|----|-----|----|
|                             |  | 6                                   | 9    | 13 | 16 | 20 | 27  | 36 |
|                             |  | Trichothecin concentration (mg./l.) |      |    |    |    |     |    |
|                             |  |                                     |      |    |    |    |     |    |
| 0.5                         | 100  | 32.0                                | 26.0 | 17 | 28 | —  | —   | —  |
| 1.0                         | 200  | 14.0                                | —    | 72 | 71 | 56 | —   | —  |
| 2.0                         | 400  | 6.3                                 | 25.0 | 55 | 58 | 74 | 91  | 57 |
| 4.0                         | 800  | 2.5                                 | 9.4  | 30 | 72 | 91 | 111 | 72 |

— Not tested.

Imperfecti, Zygomycetes and Ascomycetes, and the growth of the twenty-seven species of fungi so far examined was inhibited in some degree. Tests in the presence of a range of concentrations of trichothecin (0.13–80 mg./l., Table 8) revealed wide differences in the sensitivity of different fungi to trichothecin. The growth of *Penicillium digitatum*, much the most sensitive species examined, was completely inhibited by 0.64 mg./l. In contrast, the growth of *P. notatum*, *P. nigricans janczewskii* and *Trichoderma viride* was not completely inhibited at a concentration of 80 mg./l. Growth of *Trichothecium roseum* strain F 227 was itself partially inhibited by a trichothecin concentration of 80 mg./l.

Germination of *Penicillium digitatum* conidia was virtually suppressed by

1.25 mg./l. of trichothecin; the ED<sub>50</sub> varied from 0.80 to 0.75 mg./l. according to the age and other properties of the conidia (Table 2). The corresponding figures for *Botrytis allii* spores are 6.25 and 8.12 mg./l.

Table 8. *Effect of trichothecin on the growth of certain fungi*

The fungi were seeded on to the surface of beer-wort agar containing varying trichothecin concentrations. After 5 days' incubation at 25° growth was compared with that obtained on control plates containing no antifungal substance. No growth, 0; trace of growth, 1; slight growth, 2; good growth, 3; abundant growth (as control), 4.

| Laboratory<br>collection<br>no. | National<br>Collection<br>of Type<br>Cultures<br>no. | Organism  | Relative growth at tricho-<br>thecin concentrations (mg./l.) |    |     |      |      |
|---------------------------------|--|---|--|----|-----|------|------|
|                                 |  |   | 80   | 16 | 3.2 | 0.64 | 0.13 |
| Relative growth                 |  |   |  |    |     |      |      |
| Fungi Imperfecti                |  |   |  |    |     |      |      |
| F 153                           | 982  | <i>Aspergillus fumigatus</i> Fres.                  | 0  | 3  | 4   | 4    | 4    |
| F 160                           | 3808   | <i>A. niger</i> van Tiegh.                          | 0  | 0  | 4   | 4    | 4    |
| F 181                           | 1859   | <i>Cephalosporium longisporum</i> Petch             | 0  | 1  | 4   | 4    | 4    |
| F 182                           | 2278   | <i>Cladosporium herbarum</i> Link                   | 0  | 0  | 3   | 4    | 4    |
| F 197                           | —  | <i>Fusarium graminearum</i> Schwabe                 | 0  | 0  | 3   | 3    | 3    |
| F 180                           | 1357   | <i>Helminthosporium sacchari</i> Butl.              | 0  | 1  | 2   | 3    | 4    |
| F 17                            | —  | <i>Paecilomyces varioti</i> Bain.                   | 0  | 3  | 4   | 4    | 4    |
| F 117                           | 583  | <i>Penicillium caseicola</i> Bain.                  | 0  | 1  | 4   | 4    | 4    |
| F 229                           | 3539   | <i>P. citrinum</i> Thom                             | 0  | 1  | 4   | 4    | 4    |
| F 196                           | —  | <i>P. digitatum</i> Sacc.                           | 0  | 0  | 0   | 0    | 3    |
| F 119                           | 593  | <i>P. expansum</i> Link                             | 0  | 3  | 4   | 4    | 4    |
| F 132                           | 584  | <i>P. lilacinum</i> Thom                            | 0  | 1  | 3   | 4    | 4    |
| F 140                           | 3958   | <i>P. meleagrinum</i> Biourge                       | 0  | 0  | 3   | 4    | 4    |
| F 211                           | —  | <i>P. nigricans</i> — <i>janczewskii</i> series     | 1  | 3  | 4   | 4    | 4    |
| F 143                           | 4222   | <i>P. notatum</i> Westling                          | 1  | 4  | 4   | 4    | 4    |
| F 135                           | 983  | <i>P. oralicum</i> Currie & Thom                    | 0  | 0  | 4   | 4    | 4    |
| F 133                           | 588  | <i>P. roqueforti</i> Thom                           | 0  | 1  | 4   | 4    | 4    |
| F 234                           | 591  | <i>P. spinulosum</i> Thom                           | 0  | 0  | 4   | 4    | 4    |
| F 214                           | 6436   | <i>Stachybotris atra</i> Corda                      | 1  | 4  | 4   | 4    | 4    |
| F 5                             |  | <i>Trichoderma viride</i> Pers. ex Fries            | 1  | 3  | 4   | 4    | 4    |
| F 227                           |  | <i>Trichothecium roseum</i> Link                    | 2  | 4  | 4   | 4    | 4    |
| Ascomycetes                     |  |   |  |    |     |      |      |
| F 177                           | 4020   | <i>Chaetomium convolutum</i> Chivers                | 0  | 0  | 2   | 4    | 4    |
| F 268                           | 3411   | <i>Neurospora crassa</i> Shear & Dodge              | 0  | 0  | 0   | 4    | 4    |
| B 55                            | 742  | <i>Saccharomyces carlsbergensis</i> Hansen          | 0  | 0  | 1   | 4    | 4    |
| Zygomycetes                     |  |   |  |    |     |      |      |
| F 266                           | 1904   | <i>Mucor erechus</i> Bain.                          | 0  | 1  | 2   | 3    | 4    |
| F 264                           | 3155   | <i>Syncephalastrum racemosum</i> (Colm)<br>Schroet. | 0  | 3  | 4   | 4    | 4    |
| F 265                           | 1127   | <i>Thamnidium elegans</i> Link                      |  |    |     |      |      |

## DISCUSSION

The isolation of trichothecin and demonstration of its antifungal properties provide fairly conclusive evidence that this compound is responsible for the antagonism exhibited by *Trichothecium roseum* towards other fungi. Several other crystalline compounds have been isolated from *T. roseum* culture filtrates and will be described elsewhere, but all were devoid of antifungal or anti-

bacterial activity. Trichothecin is extracted from the filtrates by chloroform, which removes the whole of the antifungal activity. None of the fractions obtained chromatographically, with the exception of the trichothecin-containing fractions, exhibit any antifungal activity. The stability of trichothecin at normal temperatures and over a wide range of pH (Freeman & Morrison, 1948) suggests that the compound may persist for sufficiently long in nature to influence the growth of other fungi. In this connexion the frequent occurrence on dead wood of characteristic, virtually pure cultures of *T. roseum* is of interest. Concentrations of trichothecin of the order of 80 mg./l. partly inhibit growth of *T. roseum* in culture, and old cultures of the fungus slowly lose their antifungal activity.

The antifungal activity originally observed in Czapek-Dox-5 % (w/v) glucose medium proved to correspond to about 10 mg./l. of trichothecin. The bulk of the work described above was undertaken with the object of improving the yield to provide sufficient quantities of the compound for chemical analysis and degradation studies. No evidence of the mode of antifungal action of trichothecin is yet available; it has, however, been shown that its action is not inhibited by an excess of cysteine (cf. Cavallito & Bailey, 1944).

We wish to express our thanks to Mr A. J. Baillie for his assistance with the biological assays and to Miss A. McCann for assistance in the isolation of the trichothecin used in this work.

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## Outline Classification of *Bacterium* and *Staphylococcus*

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**SUMMARY:** The National Collection of Type Cultures, in its list of species maintained, uses unorthodox classifications of the genera *Bacterium* and *Staphylococcus*. For convenience in cataloguing *Bacterium* is divided into groups representing coliforms, plant pathogens, non-fermenting species and paracolons; to these is added a miscellaneous group of organisms of uncertain taxonomic position. These subdivisions are not intended to constitute a valid classification.

Coagulase-positive staphylococci, irrespective of pigment, form the species *pyogenes*; coagulase-negative strains are subdivided according to the pigment produced.

### BACTERIUM

The National Collection of Type Cultures (1948) recognizes the genera *Salmonella* and *Shigella* but includes most of the other Gram-negative intestinal bacilli in the genus *Bacterium*, which is defined in a broad sense:

*Bacterium*. Gram-negative rods, usually motile by peritrichous flagella. Grow readily on simple media and most groups are active in fermenting carbohydrates. Most species do not liquefy gelatin. Widely distributed in nature.

Type species: *Bacterium coli* Escherich.

Within the genus five groups are recognized:

*Coli-aerogenes*, active fermenters of carbohydrates, usually with the production of gas; all species ferment lactose.

*Erwinia*, similar to *coli-aerogenes* but pathogenic for plants; usually ferment salicin.

*Friedländer*, capsulated bacilli producing mucoid colonies; usually isolated from the respiratory tract.

*Non-fermenting*, fail to ferment any carbohydrates; sometimes produce alkali in milk.

*Paracolon*, active fermenters of carbohydrates; late or no fermentation of lactose.

For ease of reference we have listed the plant pathogens as a separate group, but we agree with Dowson (1939) that these organisms should be regarded as members of the main *coli-aerogenes* group, with *Bacterium coli* as the type species. Habitat is an undesirable criterion in a systematic classification, but for convenience in preparing our list we adopted it to separate coliforms from *Erwinia* and the *Friedländer* group from *aerogenes*. We hesitate to abolish the *Friedländer* group, but we do not feel justified in giving it generic rank; further study of antigenic make-up will probably decide the matter.

The paracolon group is differentiated on the one hand from the *coli-aerogenes* group by the non-lactose-fermenting or late-lactose-fermenting character of the organisms, and on the other hand from *Salmonella* and *Shigella* by differences in antigenic structure. The work of Edwards, West & Bruner (1947) has done much to show the relationship of certain paracolons (Arizona group) to the

salmonellas. The non-fermenting group is clearly differentiated from the other groups, but it is not homogeneous. Our work with this group suggests that eventually at least two genera may be justified.

In general we agree with the view of Borman, Stuart & Wheeler (1944) that the Gram-negative bacilli which we call *Bacterium*, and they call *Colobactrum*,

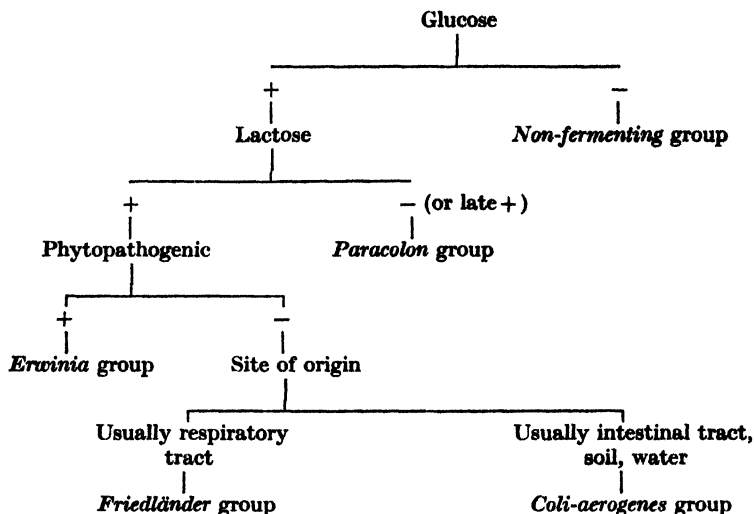


Fig. 1. Differentiation of groups within the genus *Bacterium*.

*Paracolobactrum*, *Erwinia*, and *Proshigella*, show a series of gradations which blend one into another. We differ in not yet being prepared to treat *Bacterium* (Enterobacteriaceae) as a family and subdivide it into genera. Like Wilson & Miles (1946), we recognize groups within our genus *Bacterium* and know that some may eventually be accorded generic rank.

Finally, in the National Collection of Type Cultures *List* we have included a group 'Bacterium'. The word *Bacterium* is printed here within quotes and in our *List* in roman to indicate that we do not regard this group as homogeneous or the organisms in it as necessarily related to the rest of our genus *Bacterium*. It includes species (mostly Gram-negative rods) that have been insufficiently studied or described to justify their allocation to accepted genera. In short this group consists of organisms of uncertain taxonomic status and is merely a cataloguer's dump-heap.

### STAPHYLOCOCCUS

*Staphylococcus* may be defined in one of two ways; the first includes all monomorphic Gram-positive cocci arranged in clusters, the second, more sharply circumscribed, is limited to the cocci that produce the enzyme coagulase. We propose to outline the points in favour of and against these definitions in turn.

*Staphylococcus* Ogston (1882) *emend.* Monomorphic, Gram-positive cocci less than  $1\mu$  in diameter, arranged in pairs in young cultures and in irregular clusters in older cultures. Grow readily on simple media. Under suitable conditions white, yellow,

or gold colonies are produced. Some species produce characteristic enzymes and toxins. Mainly saprophytes but sometimes pathogenic to man and animals.

Type species: *Staphylococcus pyogenes* Rosenbach *emend.*

The characteristic enzymes are staphylocoagulase and fibrinolysin. The coagulase test should be standardized and carried out with human plasma (Gillespie, 1948; Williams & Harper, 1946); we stress this because recent work suggests that different mechanisms take part in the reaction when plasma of different species are used (Smith & Hale, 1944). A genus so defined would be acceptable to most bacteriologists; however, on grounds of pleomorphism and pigment it would exclude organisms such as *Staphylococcus flavo-cyaneus* (Knaysi, 1942). The chief and formidable objection to this definition is that the borderline between this genus and *Micrococcus* is ill-defined, because it rests on the diameter of the cocci and therefore on uniformity in size and shape. Most micrococci have cells  $1\mu$  or more in diameter and show variation in size, but cell size may vary so much under different cultural conditions that it is impossible to make a precise dividing line between the two genera.

The second definition we considered was simpler:

*Staphylococcus* Ogston (1882) *emend.* Gram-positive cocci that produce an enzyme capable of clotting human plasma.

Type species: *Staphylococcus pyogenes* Rosenbach *emend.*

Such a genus is neat and compact; it consists of organisms that are all closely related serologically, sharing a series of specific antigens (Cowan, 1939; Christie & Keogh, 1940; Hobbs, 1948). Furthermore, most strains of this genus are susceptible to one or more of a series of staphylococcal bacteriophages (Wilson & Atkinson, 1945). Most strains are capable of producing one or more of the recognized staphylococcal toxins, and all are regarded as potentially pathogenic (Cruickshank, 1937; Fairbrother, 1940; Miles, Williams & Clayton-Cooper, 1944).

It may be objected that the definition excludes a group of coagulase-negative, monomorphic, Gram-positive cocci that are normally accepted as staphylococci. This group would have to be placed either in a new genus created for them or in the genus *Micrococcus*. For our part we should not object to enlarging the genus *Micrococcus* to include this group and should like to have defined *Staphylococcus* to exclude it; but in preparing the N.C.T.C. List we had to bear in mind that it would be read by scientists of all kinds and that all must be able to recognize the species named. As a consequence our genus *Staphylococcus* had to conform to the first definition given above and include both coagulase-positive and coagulase-negative forms. While this definition is now generally acceptable we think that systematists, bearing in mind the importance of antigenic relationships, may ultimately adopt a definition closer to our second but with another criterion in place of coagulase, such as sensitivity to a polyvalent staphylococcal bacteriophage.

Our genus *Staphylococcus*, conforming to the first definition, is divided into five species, whose differentiation is shown in the second schema (Fig. 2).

Coagulase for human plasma is one of the most stable characters of staphy-

lococci, and the primary subdivision is made on the production of this enzyme; all positive strains, irrespective of pigment, are named *Staphylococcus pyogenes*. Pigment production is regarded as having only secondary importance, and we do not recognize the varieties *albus*, *aureus* and *citreus* of the genus *pyogenes*. These varieties cut across the more stable characters such as antigenic structure and phage type.

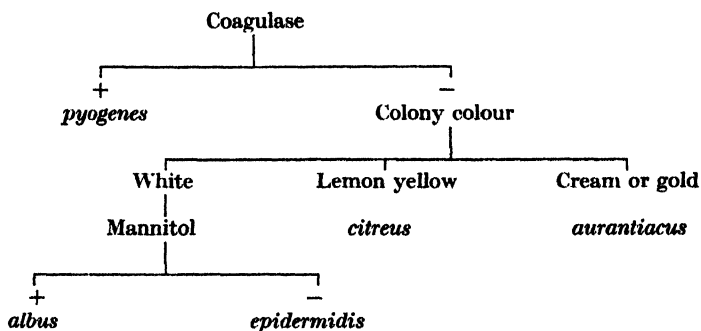


Fig. 2. Differentiation of species within the genus *Staphylococcus*.

In the absence of better differential characters pigmentation is of some use in the subdivision of the coagulase-negative group, and we recognize the species *aurantiacus* and *citreus* on the ground of pigment alone. White-coloured strains are divided into two species by the fermentation of mannitol; our species *epidermidis* agrees largely with Andrewes & Gordon's (1907) *Staphylococcus epidermidis-albus* and with Cowan's (1988) *Staphylococcus epidermidis*; to the mannitol-fermenting strains we have given the name *Staphylococcus albus*, which agrees with the species so named of Bergey, Breed, Murray & Hitchins (1939).

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## Further Observations on the Differential Inhibition of Coliform Bacilli and Rough Variants of Intestinal Pathogens

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**SUMMARY:** The pattern of differential inhibition of Gram-negative bacilli of intestinal origin depends not only upon a synergy of bile salts and electrolytes of the Hofmeister series but also upon aerobiosis and the nature of the surface upon which they are grown (Brodie, 1948). Identical patterns of inhibition are obtained if shallow-layer fluid cultures are employed. The media under discussion possess lethal qualities which appear to be modified by a process akin to adsorption and the results obtained with solid media to depend in part upon the physical nature of the gel.

Electron microscope studies suggest that bile salts alter the permeability of the cell-wall, facilitating the entry of electrolytes. The resultant swelling or disruption of cells exposed to bile-salt + electrolyte mixtures and subsequently to distilled water is a measure of their electrolyte content. The ease with which organisms can be inhibited in the media seems to be directly related to their altered cell-wall permeability. The observations direct attention to physiological differences between rough and smooth variants of a single culture. They indicate that one factor upon which such differences depend is the greater permeability of the rough variant to electrolyte in the presence of bile salts.

A further attempt has been made to elucidate the mechanism of differential inhibition in media containing bile salts and electrolytes. Brodie (1948) showed that agar and silica gels gave consistent results, whereas the effect of gelatin was less certain. Agar and silica gels on setting extrude water by syneresis whereby their surfaces are 'wetted', but gelatin gels may have a 'dry' surface. To obtain the requisite patterns of differential inhibition, a 'wet' surface appears to be essential. Assuming that the fluid extruded by the agar and silica gels contains all the soluble constituents of the medium in their original concentrations, then it should be possible to reproduce the same patterns of inhibition by growing the organisms in corresponding fluid media. Furthermore, if, as indicated by previous work, aerobiosis is essential, then only by exposure of the organisms in shallow-layer cultures should the differential effect be obtained. Since the gelatin-containing medium remains fluid at 37°, the behaviour of the organisms in the presence of gelatin in the fluid state along with the other constituents was also open for study. With the *Sonne III* variants the inhibitory mechanism shows a selectivity which is partly lethal and differs in degree for either variant.

### *Media and technique*

Unless otherwise indicated, all media were fluid and contained sodium tauroglycocholate (British Drug Houses Ltd., B.P.C. standard), sodium chloride and peptic digest of serum (Brodie, 1942). Each 6 ml. of complete medium

contained: 1.5 ml. peptic digest of serum; 0.6 ml. of 5 % (w/v) sodium tauroglycocholate; 0.6 ml. of 3.6 % (w/v) sodium chloride; the requisite volume of electrolyte solution to give a known molar concentration; sufficient double glass-distilled water to bring the final volume to 6 ml. The reaction of all media was adjusted to pH 7.6. Media were steamed for 30 min. before use. Where available, 'Analar' reagents (British Drug Houses, Ltd.) were used.

Sufficient of a 24 hr. agar culture to produce minimal visible turbidity was transferred on a chrome-iron wire loop to 9 ml. of sterile distilled water. After vigorous mixing, 0.1 ml. of this suspension was thoroughly dispersed in a bottle containing 90 ml. of sterile distilled water; from this 0.5 ml. was inoculated into the tube ( $6 \times \frac{3}{4}$  in.) containing the experimental medium. Thereupon 1 ml. from the inoculated tube was mixed in a Petri dish with 4 ml. of melted C.C.Y. agar (Gladstone & Fildes, 1940), allowed to set and incubated for 24 hr. at 37°. The colony count at the end of this period was assumed to be the number of viable organisms/ml. of inoculated medium. All Petri dishes used had an internal diameter of  $1\frac{3}{4}$  in. and an internal depth of  $\frac{3}{4}$  in. Further counts of the viable organisms remaining in the experimental medium were made in a similar fashion at intervals. Any departures from these methods are noted in the text.

*Organisms studied.* The organisms used were the same as in the previous work, namely: SR, a rough variant of a recently isolated *Sonne III* bacillus; SS, a smooth variant from the same parent strain; C1, *Bacterium coli communes* from human faeces; C2, *Bacterium coli communius* from human faeces; A1, *Aerobacter* sp. from milk; A2, *Aerobacter* sp. from water.

*Standard patterns of inhibition.* The patterns of inhibition to which reference will be made are: (a) 'partial differentiation', which means that SR, SS, A1 and A2 grew but C1 and C2 failed to grow; (b) 'complete differentiation', indicating that all strains, except SS, failed to grow.

## RESULTS

### *Shallow and deep cultures*

Having inoculated the 6 ml. tubes of media and prepared the viable inoculum plates, 1 ml. volumes were spread in Petri dishes to give shallow cultures. These were incubated for 24 hr. in air saturated with water vapour at 37°. The 4 ml. volumes remaining were left in the tubes and also incubated for 24 hr. at 37°. Counts were then made from both shallow and deep cultures. Partial and complete differentiations occurred only with the shallow cultures. In the presence of 0.5 % tauroglycocholate in shallow culture, partial differentiation resulted with 0.057 M ammonium citrate, 0.066 M potassium citrate, 0.1 M sodium citrate, 0.38 M sodium sulphate and 0.53 M sodium chloride; and complete differentiation with 0.1, 0.12, 0.2, 0.398 and 0.66 M concentrations of these salts respectively. These molar concentrations are identical with those required to produce the same differentiation on the surface of bile-salt agar media (Brodie, 1948).

*Media containing gelatin*

Similarly compounded media containing 15% (w/v) gelatin were tested, using 0.1 and 0.2 M sodium citrate as the electrolyte and both shallow and deep culture. The shallow-layer cultures were incubated at 37° without any precautions being taken to saturate the air with water vapour. The viable inoculum counts in this instance were made from distilled water (see next section). Partial differentiation was achieved with 0.1 M and complete differentiation

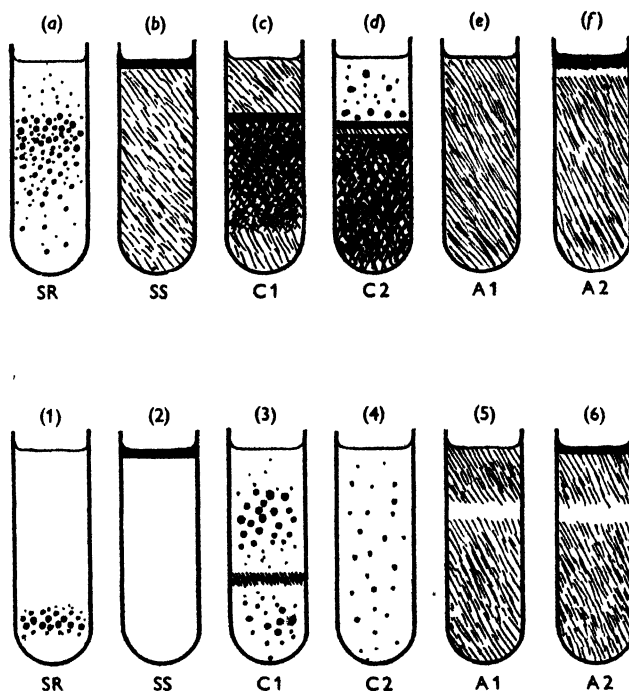


Fig. 1. Diagrammatic representation of 8-day growths of rough variant of *Sonne III* (SR), smooth variant of *Sonne III* (SS), *Bact. coli communes* (C1), *Bact. coli communis* (C2), *Aerobacter* sp. from milk (A1) and *Aerobacter* sp. from water (A2) in 15% gelatin bile salt electrolyte media; (a)–(f) contain 0.1 M sodium citrate and (1)–(6) 0.2 M sodium citrate. Densities of growth are indicated by corresponding densities of shading.

with 0.2 M sodium citrate only in the shallow cultures. In the deep cultures all six strains grew in 0.1 M and all but C2 grew in 0.2 M sodium citrate. Fluid gelatin medium in shallow layers gave the same result as fluid media and agar surface cultures.

The deep cultures, having been tested for growth after 24 hr. at 37°, were allowed to set and incubated a further 8 days at 22°; duplicate tubes heavily inoculated from thick distilled water suspensions were set up as controls, these being incubated directly at 22° for 8 days. The results were substantially the same with or without preliminary incubation at 37°. However, with the higher concentration of citrate the air tolerance of the organisms, with the exception of SS, was depressed (Fig. 1). It was previously observed (Brodie, 1948) that

the differential effects obtained on the surface of agar gels were not reproduced using gelatin gels. On this occasion we have failed to corroborate the previous findings with gelatin. At present we cannot offer any explanation of this although both tests have been repeated several times.

### Viability

During the experiments with fluid media it was noted that, whereas the initial turbidities in the 9 ml. distilled water tubes were of the same approximate densities, the counts made from the media varied markedly. This was most notable with SR, where some of the counts were nil. Distilled water and

Table 1. *Numbers of Sonne III rough (SR) and Sonne III smooth (SS) variants surviving in deep and shallow cultures in peptic digest of serum broth containing 0.5 % (w/v) sodium chloride, 0.5 % (w/v) sodium tauroglycocholate and various concentrations of sodium sulphate*

| Strain | Sodium sulphate (M) | Distilled water control (0-15 min.) | Deep culture after (min.) |      |      |      | Shallow culture after (min.) |      |      |      |
|--------|---------------------|-------------------------------------|---------------------------|------|------|------|------------------------------|------|------|------|
|        |                     |                                     | 0                         | 5    | 10   | 15   | 0                            | 5    | 10   | 15   |
|        |                     |                                     | Counts                    |      |      |      | Counts                       |      |      |      |
| SR     | 0.33                | 9000                                | 1000                      | 60   | 30   | 24   | 1100                         | 280  | 86   | 44   |
| SR     | 0.398               | 9000                                | 300                       | 7    | 0    | 0    | 430                          | 130  | 40   | 20   |
| SS     | 0.33                | 4000                                | 2300                      | 1800 | 1500 | 1100 | 2400                         | 2400 | 2400 | 2400 |
| SS     | 0.398               | 4000                                | 1500                      | 1000 | 775  | 775  | 2400                         | 2400 | 2400 | 2400 |

the media were therefore examined. The electrolyte used was sodium sulphate. Suspensions of SR and SS were prepared as before and each inoculated into 6 ml. tubes of distilled water and also into bile-salt media containing 0.33 and 0.398 M-sodium sulphate. Counts of viable organisms were made immediately, and at 5, 10 and 15 min. intervals. The counts from the distilled water tubes remained constant throughout the 15 min. period, but the two media exhibited lethal action. The experiments recorded in Table 1 indicate that the media exhibit a lethal effect which depends on (a) the variant exposed, (b) the access of air and (c) the concentration of added electrolyte. We realize that, owing to the susceptibility of rough strains to salt agglutination, the diminution in colony count with SR might be partly accounted for thereby. The suspensions were therefore prepared to contain relatively few organisms—9000/ml.—to diminish spontaneous aggregation. Viewed in relation to the findings as shown by culture and observation with the electron microscope, the balance of evidence is that the medium was lethal.

### Relation of inoculum to lethal effect

It was decided to try the effect of adding known numbers of SS cells to the medium containing 0.398 M sodium sulphate and to test the medium after each exposure for any alteration in its lethal effect. A batch of the fluid medium containing 0.5 % tauroglycocholate and 0.398 M sodium sulphate was divided

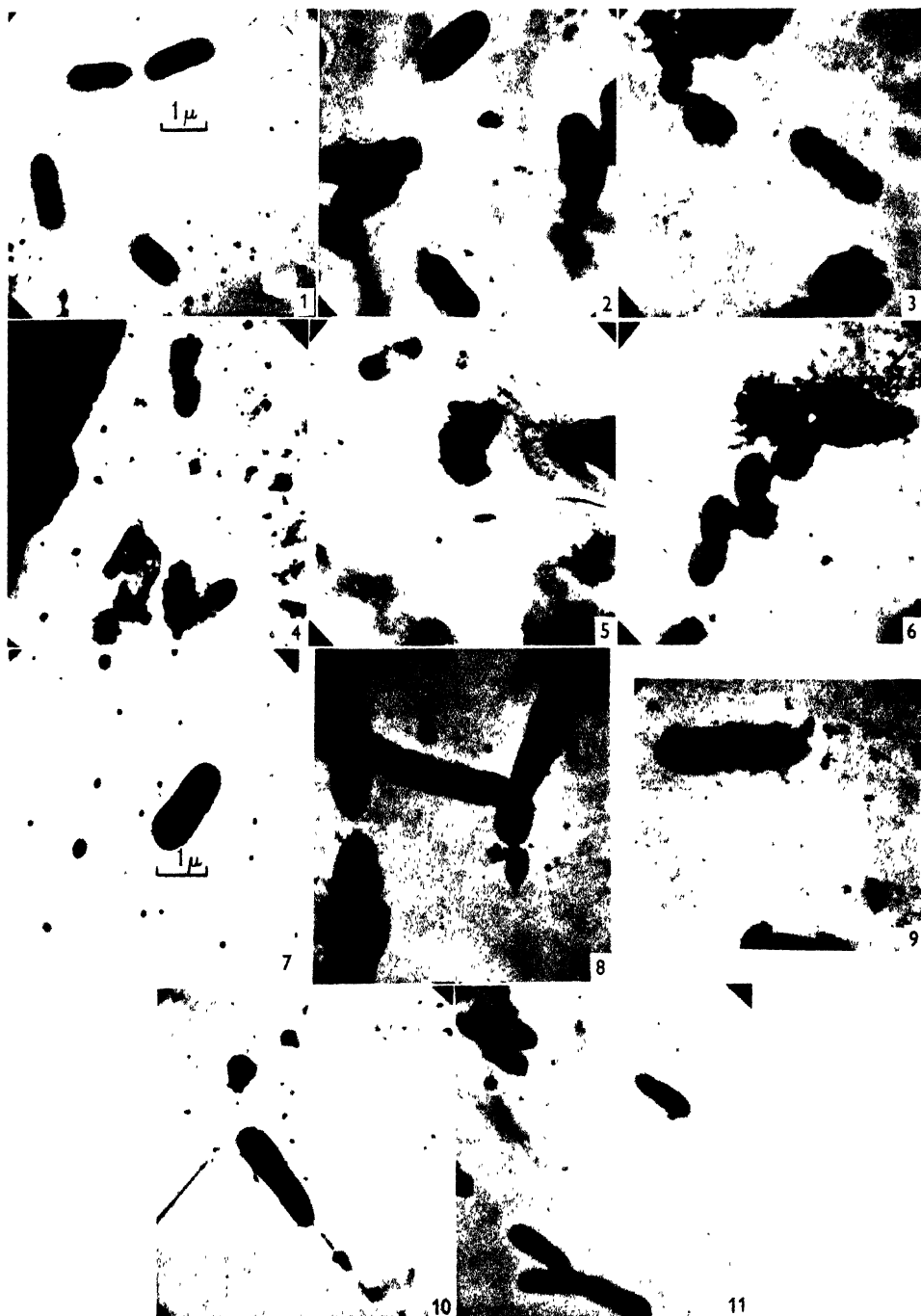
into parts A and B. To part A, packed washed living SS cells were added to yield, as calculated by Brown's scale, a concentration of 45,000 million organisms/ml. After thorough mixing and 5 min. exposure, the organisms were removed by high-speed centrifugation. The supernatant was collected, its volume measured, then heated at 65° for 45 min. to sterilize. This procedure was repeated but with a small dose of organisms, the number being determined by counting from distilled water, and the number of survivors after 5 min. exposure to the medium counted. Finally, a third addition of organisms was made, similarly estimated and the effect of 5 min. exposure determined. Part B of the medium was treated in the same way except that all three doses of added organisms were small.

Part A, after treatment with the massive dose of cells, was no longer lethal for the subsequent inocula: of 11,500 and 12,200 organisms/ml. added as the second and third inocula, 11,400 and 12,000/ml. survived the 5 min. exposures, a survivor rate of almost 100 %. Part B, with initial inocula of 6250, 14,000 and 12,500 organisms/ml., yielded 8000, 10,000 and 9500 survivors/ml. respectively; survival rates of 48, 71 and 76 %. Part A, now rendered devoid of lethal effect for strain SS, was tested for lethal effect on SR and was found to be as lethal to SR as the untreated medium; this was equally true in respect of 5 min. exposure and of 24 hr. exposure in shallow culture at 37° with initial inocula of 12,000/ml. in both. The possibility of salt agglutination must, however, be borne in mind.

#### *Observations with the electron microscope*

Preparations were examined with the electron microscope (Metrovick E.M. 2) at 50 kV. accelerating potential and a beam-current of 0.2 ma., employing minimum magnification which, with the length of specimen holder employed, was approximately 6000 diameters. Distilled water suspensions of SR and SS cells were made and examined after vacuum drying on collodion films. These served as the basis of comparison with the other preparations which were made by suspending washed SR and SS organisms in 0.8 M and 0.898 M sodium sulphate bile-salt media for 80 min. at 37°, removing them by centrifugation and resuspending them in distilled water prior to examination as above.

Photographs of preparations are shown on Plate 1. The SS strain after treatment as above is little altered (Pl. 1, figs. 7-10). The SR strain after similar exposure (figs. 1-5), shows marked swelling and internal distortion with 0.88 M sodium sulphate + bile salt (fig. 4); with 0.898 M sodium sulphate + bile salt all but a few are burst, only the cell wall remaining (fig. 5). Exposure of these organisms to bile salt or electrolyte alone produced no such effects (figs. 2, 3). Exposure of the coliform bacilli (fig. 6) and the *Aerobacter* sp. (fig. 11) to bile salt + 0.898 M sodium sulphate caused swelling and internal distortion of these organisms similar to that noted with the SR strain treated with 0.88 M sodium sulphate + bile salt. Throughout this investigation, the serological characters of the SR and SS strains were checked by agglutination.



Figs. 1-11



The authors wish to thank Prof. W. J. Tulloch for advice and criticism and Prof. G. D. Preston of the Physics Department and his staff for permission to use the electron microscope and for assistance in its use. The Department of Physics is indebted to the Department of Scientific and Industrial Research for the loan of a Metropolitan-Vickers type E.M. 2 electron microscope.

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## EXPLANATION OF PLATE

Electron micrographs of strains SR, SS, C2 and A2 made from distilled water suspensions, either A, direct from agar cultures, or B, after exposure for 30 min. at 37° in fluid medium containing (1) 0.5 % tauroglycocholate only, (2) 0.398 M sodium sulphate only, (3) 0.5 % tauroglycocholate and 0.33 M sodium sulphate or (4) 0.5 % tauroglycocholate pure 0.398 M sodium sulphate. Magnifications are indicated by the 1  $\mu$  mark on figs. 1 and 7.

Fig. 1. Strain SR treated as A. Fig. 2. SR after B(1). Fig. 3. SR after B(2). Fig. 4. SR after B(3), swelling and internal distortion are evident. Fig. 5. SR after B(4), one organism shows swelling and internal distortion while the others are burst, only their cell-membranes remaining. Fig. 6. C2 after B(4), showing swelling and internal distortion. Fig. 7. SS prepared as A. Fig. 8. SS after B(1). Fig. 9. SS after B(2). Fig. 10. SS after B(4), showing neither swelling nor distortion (cf. Fig. 5). Fig. 11. A2 after B(4) shows some swelling and internal distortion.

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## The Streptococci of Group D; the Serological Grouping of *Streptococcus bovis* and Observations on Serologically Refractory Group D Strains

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**SUMMARY:** The concentration of the group-specific substance in hydrochloric acid extracts by precipitation with ethanol has facilitated the serological identification of *Streptococcus bovis* and certain other serologically refractory streptococci as members of group D. The notorious difficulty of preparing potent group sera for *Str. bovis* was overcome by immunizing rabbits with organisms shaken with an abrasive in a magnetic vibrating machine. The relationship of *Str. bovis* to certain other streptococci and the chemical nature of the specific substance of group D are discussed.

In a serological grouping of haemolytic streptococci from various sources Lancefield (1938) placed in group D a small collection of streptococci isolated from cheese, and Sherman (1938) identified them by biochemical characters as *Streptococcus zymogenes*. Although Lancefield's classification was originally confined to haemolytic members of the genus, it soon became evident that many non-haemolytic streptococci had group-specific substances in common with haemolytic varieties.

It has been established that the intestinal streptococci, *Str. faecalis* and its variants *zymogenes* and *liquefaciens*, and also *Str. durans* fall into group D (Sherman, 1938; Graham & Bartley, 1939; Shattock & Mattick, 1943; Shattock, 1945).

On the other hand, *Str. bovis*, a *Streptococcus* conspicuous in cow-dung and common in milk, has proved difficult to place serologically, although biochemically it is a well-defined species. Raffinose-fermenting streptococci were early recognized as characteristic of the bovine intestine, and Orla-Jensen (1919) introduced the specific name, *Str. bovis*, on the basis of fermentation tests. The studies of Ayres & Mudge (1923) on streptococci from bovine mouths and intestines and of Sherman & Stark (1931) on streptococci growing at high temperatures have provided valuable additional information on this species.

Sherman & Stark (1931) ascribed to *Str. bovis* the following characteristics: blood not haemolyzed; litmus milk not reduced before curdling; growth at 45° but not at 10 or 50°; arabinose, glucose, maltose, lactose, sucrose, raffinose, starch and salicin, and sometimes mannitol and inulin fermented, but glycerol not; ammonia not produced from peptone. Sherman (1938) also observed that *Str. bovis* does not grow in the presence of 6.5 % NaCl or 0.1 % methylene blue or at pH 9.6. Although Sherman (1937) recorded that *Str. bovis* survived 60° for 80 min., strains isolated in this laboratory and tested by the technique described here have consistently failed to pass this test although they withstand 60° for 15 min. (Shattock & Mattick, 1948).

*Str. bovis* has many of the characteristics of group D streptococci, but

nevertheless is readily distinguished biochemically from *Str. faecalis* and its variants, and some workers have preferred to separate it from the *Enterococcus* group. It has been reported (Sherman, 1938; Shattock, 1944) that whereas many strains of *Str. bovis* give negative or equivocal results with the usual Lancefield technique, about 50 % react clearly with group D sera. In a preliminary note Shattock (1948), on serological grounds, assigned *Str. bovis* to group D, and experimental details establishing the serological identity of *Str. bovis* are presented here.

In addition to *Str. bovis*, streptococci are frequently encountered having cultural reactions similar to, and sometimes identical with, the species within group D, but which fail to react with potent group D sera. For some years such equivocal strains have been collected from a variety of sources. The serological grouping of these strains is also dealt with in this paper.

## MATERIALS AND METHODS

### *Biochemical and cultural methods*

**Haemolysis.** Brown's (1919) technique was followed using, instead of a veal infusion base for the horse-blood plates, a nutrient agar consisting of 2 % agar, 1 % Evans's peptone, 1 % Lemco and 0.5 % NaCl.

**Reducing properties.** Complete or almost complete reduction of litmus milk in 24 hr. at 37° was recorded as 'strong reduction'.

**Gelatin liquefaction.** Stab cultures were incubated at 22° and read after 14 days.

**Carbohydrate fermentations.** Peptone water containing 0.5 % of the test sugars, added after sterilization, with litmus as indicator, were inoculated with 1 loopful (4.0 mm.) of an 18 hr. broth culture, and incubated at 37°. Results were read after 5 days' incubation.

**Growth at 45°.** Tubes of glucose (1 %) Lemco broth seeded with 2 loopfuls (4.0 mm.) of an 18 hr. culture were incubated in a water-bath at  $45 \pm 0.1^\circ$ . Tubes were examined for growth after 24 hr.

**Growth at pH 9.6.** The buffered liquid medium described by Shattock & Hirsch (1947) was used.

**Survival at 60° for 30 min.** Two loopfuls (4.0 mm.) of an 18 hr. culture were inoculated into tubes of glucose (1 %) Lemco broth held in a water-bath at  $60 \pm 0.10^\circ$  for 30 min., cooled immediately, incubated at 37° for 24 hr. and examined for growth.

### *Serological methods*

**Routine preparation of antisera for group D.** The preparation of potent sera for group D is notoriously troublesome. As with other Lancefield groups the choice of immunizing strain is important. Over a period of years many strains of all species within group D have been used for this purpose, and, though group sera have been prepared successfully from all the established species and variants within the group, a strain of *Str. durans* (98D) kindly supplied by Prof. Sherman has so far produced the most consistently potent serum. Though

it is possible to produce a group D serum with formalin or heat-killed organisms a more reliable method is that of Shattock & Mattick (1948) using acetone-extracted ground organisms. The 24 hr. growth from 4–5 l. of glucose Lemco broth, incubated at 37°, is centrifuged, the organisms resuspended in approximately 100 ml. acetone and extracted at room temperature for 4–5 hr. in a mechanical shaker revolving approximately 70 times/min. The acetone is discarded and extraction once repeated. The extracted organisms are dried *in vacuo* over  $P_2O_5$  and finally ground in a ball mill until microscopic examination shows that very few intact cocci remain. The resulting powder can be stored indefinitely in a vacuum desiccator over  $P_2O_5$  at room temperature. One batch of powder stored for 8 years still induced potent group D sera, and a good group N serum was obtained with a similar preparation 9 years old.

For injection the powder is suspended in 0.85 % NaCl to give an opacity equivalent to Brown's tube no. 7 (Burroughs Wellcome and Co., Red Lion Square, London, W.C. 1). Rabbits are injected every 3–4 days starting with 0.25, 0.5 and then 1.0 ml. doses. It is well known that the individual rabbit response varies enormously and a potent specific group serum may be produced after 6–8 injections, but more often it is necessary to give two or three series of injections before a satisfactory serum is obtained. A rabbit which responds well is invaluable and may be rested, given one or two boosting injections and bled at intervals until eventually non-specific antibodies make their appearance. One rabbit was a source of potent and specific group D serum for 3 years. Specificity and group antibody potency are checked and sera are stored in the cold without preservative.

Although this technique has been very satisfactory for the routine production of group D sera and has been used successfully with *Str. faecalis* and its variants, and with *Str. durans*, it has not proved suitable with *Str. bovis*. It was thought that the comparatively long grinding in a ball mill might destroy the group substance, apparently present only in small amounts in *Str. bovis*, and other methods of rupturing the organisms were accordingly tried.

*Preparation of group sera for Str. bovis.* Strains of *Str. bovis* were grown for 24 hr. at 37° in glucose Lemco broth, the organisms removed by centrifuging and resuspended in 0.85 % NaCl to give an opacity equivalent to Brown's tube no. 10. Ten ml. of the suspension were then transferred to a cylindrical glass vessel of 20 ml. capacity containing 0.3–0.5 g. washed and sterilized carborundum (grade 100). The vessel was shaken in a magnetic shaker (Mickle, 1948) having a frequency of 50/sec. and an amplitude of *c.*  $\frac{1}{2}$  in. Suspensions of the three strains of *Str. bovis* and of *Str. durans* 98D became virtually sterile when shaken in this way for 90 min.

Sera were first prepared by giving, at 3–4-day intervals, four graded injections of formalin-killed suspensions followed by three 1 ml. doses of organisms shaken with carborundum for 60 min. and freshly prepared for each injection. In a second experiment with the same strains of *Str. bovis* the preliminary injections with formalin-killed suspensions were omitted. Potent group sera were produced by giving, at intervals of 3–4 days, four 1 ml. doses of organisms shaken with carborundum for 90 min. and freshly prepared for each inocula-

tion. In both experiments the group D strain, *Str. durans* 98D, used in this laboratory for the routine production of group D sera, was included as a control and by both treatments potent and specific group D sera were obtained.

*Extracts.* Of the various methods of extraction tried (Shattock & Mattick, 1948) Lancefield's (1933) technique gave the most reliable results for group D.

Group D cocci are grown in 50 ml. glucose Lemco broth at 37°, preferably for 48 hr.; the extra 24 hr. incubation results in more potent extracts. The centrifuged organisms are extracted for 10–12 min. in a boiling water-bath with 1.5 ml. 0.05 N-HCl made up in 0.85 % NaCl, and neutralized with little delay; extracts stored overnight in the ice-chest before neutralizing may deteriorate considerably. In our experience the deterioration of the group-specific substance by storage in weak acid is not a characteristic of other groups; unneutralized HCl extracts of group B can be stored at room temperature overnight without losing potency.

For some reason not fully understood, HCl extracts of group D streptococci are very sensitive to slight differences in their preparation. Different brands of peptone vary greatly in their suitability. With some peptones, though growth is good, extracts are cloudy or opalescent and unsuitable for ring tests. Evans's peptone (Evans Medical Supplies Ltd.) proved the most reliable brand for this work.

Mention has already been made of the difficulty, even with potent group D sera, of grouping many of the streptococci having cultural and biochemical reactions very similar to, or even identical with, the various members of group D. *Str. bovis* in particular has proved troublesome. The failure of these strains to give potent HCl extracts is not necessarily associated with weight of growth, and it was thought that these refractory strains might be poor in group substance.

Lancefield (1928), working with group A, separated the protein-type substance from the carbohydrate group substance by precipitation with ethanol and concentrating the group substance in the supernatant fluid. Following this technique with HCl extracts of typical group D strains, it was unexpectedly found that ethanol precipitated both the type and group substances, while the supernatant apparently contained no serologically precipitable material. Preliminary work indicates that the group-specific substance in these strains is a protein, and if this is confirmed some of the difficulty in dealing with refractory group D strains may be explained. Foley & Wheeler (1945), in dealing with strains from pathological sources found that of four group D strains of different serological types the group substance was of a protein nature in three and carbohydrate in one, but they did not discuss this anomalous observation.

The precipitation of the group substance from HCl extracts by ethanol provided a routine method for dealing with refractory strains. If HCl (Lancefield) extracts do not react with group D sera, the extracts after the addition of 4 vol. ethanol are allowed to stand overnight in the ice-chest (a small crystal of sodium acetate facilitates precipitation). The resulting precipitate from 1.5 ml. of HCl extract is well mixed with 0.3–0.5 ml. of 0.85 % saline, and the

small quantity of insoluble material removed by centrifuging. The clear supernatant (fraction A) contains the concentrated group substance and may be used for the precipitin test. The specificity of fraction A prepared from many strains of group D has been checked against sera for other groups, and similar ethanol precipitates from strains of other groups (including group N and *Str. thermophilus*) consistently failed to react with group D sera.

**Precipitin test.** A small quantity of serum is introduced with a Pasteur pipette into a tube with an internal diameter of 3 mm., the extract is layered on top and allowed to stand at room temperature. The junction of the two fluids is examined for ring formation against a standard diffused light. With a very potent serum the reaction takes place in a few seconds, but 10–15 min. may be required with a weak serum. Each batch of serum when harvested is tested for approximate reaction time against a selection of group D strains of different serological and biochemical types, and the reaction period for unknown strains is arranged accordingly. A serum giving a well-defined ring within 5 min. is satisfactory.

**Absorption tests.** Organisms for absorption are grown in glucose Lemco broth at 37° for 48 hr. The cells are centrifuged off, washed once in 0.85 % NaCl, resuspended in saline to give a density equivalent to 10 times Brown's opacity tube no. 10, killed by heating at 60° for 60 min. and then packed by centrifuging. When suspended in undiluted antiserum the absorbing suspension has 50 times the opacity of Brown's tube no. 10. Absorption was carried out at 37° for 1 hr., followed by refrigeration overnight before removing the cocci by centrifuging. The absorbed sera are tested for precipitins by the ring test and the results checked by Lancefield's (1938) technique with various dilutions of extract, reading the reaction after 2 hr. at 37° and after standing in the ice-chest overnight.

## RESULTS

### *The serological grouping of Streptococcus bovis*

Reference has been made to difficulties in classifying *Str. bovis*. Although some affinity with group D streptococci has been recognized, HCl extracts of typical strains of *Str. bovis* have often given anomalous results with sera for group D. By concentrating the group substance in fraction A, refractory strains of *Str. bovis* will react with a group D serum. At least forty-five strains having the biochemical characteristics of *Str. bovis* were examined by this technique, and none failed to give a clear-cut specific reaction with group D sera.

Group-specific sera were prepared against two strains of *Str. bovis*, 'Pearl 11' and 'Rosalie 20', isolated from cow-dung and having the typical species characteristics. That sera produced from these strains of *Str. bovis* did possess group and not merely type antibodies was demonstrated by their precipitation with extracts of twelve heterologous group D strains comprising at least three distinct serological types within group D. They were: *Str. faecalis*, two strains; var. *zymogenes*, four strains; var. *liquefaciens*, two strains; *Str. durans*, two strains; and *Str. bovis*, two strains. The specificity of the sera was established

by testing against HCl extracts of representatives of each of the Lancefield groups A-N and with a strain of a *Staphylococcus* rich in the non-specific nucleoprotein fraction common to all streptococci and staphylococci (Lancefield, 1925).

*Reciprocal absorption tests*

The serological identity of *Str. bovis* with group D was confirmed by reciprocal absorption tests with the sera for *Str. bovis* and sera prepared from the strain of *Str. durans* used in this laboratory for the routine production of group D sera.

*Absorption of a group D serum with Str. bovis.* A potent and specific group D serum prepared against *Str. durans* 98D was absorbed with three strains of *Str. bovis*: 'Pearl 11', 'Rosalie 20' and 'Campion 11'. The absorbed sera were tested for group D antibodies with an extract of *Str. faecalis* 'C and G', which was chosen as being of a different serological type from the group D strain 98D and for this reason unlikely to confuse the issue by reacting with type antibodies.

Table 1. *Absorption of group D antibodies by Streptococcus bovis*

| Extract                        | Group D serum |            |              |              |
|--------------------------------|---------------|------------|--------------|--------------|
|                                | Unabsorbed    | Absorbed   |              |              |
|                                |               | 'Pearl 11' | 'Rosalie 20' | 'Campion 11' |
| <i>Str. faecalis</i> 'C and G' | +             | —          | —            | —            |
| <i>Str. bovis</i> 'Pearl 11'   | +             | —          | —            | —            |
| <i>Str. bovis</i> 'Rosalie 20' | +             | —          | —            | —            |
| <i>Str. bovis</i> 'Campion 11' | +             | —          | —            | —            |

Precipitation was observed as ring tests and confirmed by incubation of various concentrations of extract against the same volume of serum (Lancefield, 1933). Results summarized in Table 1 show that group D antibodies were completely absorbed by all three strains of *Str. bovis*. In a parallel control test a group B streptococcus failed to absorb any antibodies.

*Absorption of Str. bovis sera with group D streptococci.* Sera prepared against 'Pearl 11' and 'Rosalie 20' were absorbed with *Str. faecalis* 775 and *Str. durans* 98D, and the absorbed sera tested with an extract of the group D strain *Str. faecalis* var. *zymogenes* 'Black'. Again there was complete absorption of group antibodies (Table 2). A control absorption test with a group B *Streptococcus* was negative.

Table 2. *Absorption of group sera for Streptococcus bovis by group D streptococci*

| Extract  | Serum 'Pearl 11' |          |      | Serum 'Rosalie 20' |          |      |
|--|------------------|----------|------|--------------------|----------|------|
|  | Unabsorbed       | Absorbed |      | Unabsorbed         | Absorbed |      |
|  |                  | 775      | 98 D |                    | 775      | 98 D |
| <i>Str. faecalis</i> var. <i>zymogenes</i> 'Black' | +                | —        | —    | +                  | —        | —    |
| <i>Str. faecalis</i> 775                           | +                | —        | —    | +                  | —        | —    |
| <i>Str. durans</i> 98 D                            | +                | —        | —    | +                  | —        | —    |

*Serological experiments with refractory group D streptococci*

Reference has been made to streptococci resembling, biochemically, the recognized species and variants within group D but which fail to react with potent group D sera by the usual Lancefield technique. The technique used to make potent fraction A extracts of *Str. bovis* was applied to a collection of these aberrant strains isolated from human faeces, cow-dung, gut of fly, water, cheese and dried egg.

Table 3. *The physiological characters of group D strains reacting with concentrated extracts (fraction A), but not with crude HCl extracts*

| Species or variants                           | No. of strains | Haemolysis | Gelatin liquefaction | Strong reduction of litmus milk | Mannitol      | Sucrose | Raffinose | Survival at 60° for 30 min. | Growth at 45° | Growth at pH 9.6 |
|---|----------------|------------|----------------------|---------------------------------|---------------|---------|-----------|-----------------------------|---------------|------------------|
| <i>Str. faecalis</i>                          | 10             | -          | -                    | +                               | +             | +       | -         | +                           | +             | +                |
| <i>Str. faecalis</i> variants                 | 3              | -          | -                    | -                               | +             | -       | -         | +                           | +             | +                |
|   | 2              | -          | -                    | -                               | +             | +       | +         | +                           | +             | +                |
|   | 1              | -          | -                    | +                               | +             | +       | +         | +                           | +             | +                |
|   | 6              | -          | -                    | -                               | +             | +       | -         | +                           | +             | +                |
| <i>Str. faecalis</i> var. <i>liquefaciens</i> | 3              | -          | +                    | +                               | +             | +       | -         | +                           | +             | +                |
| <i>Str. faecalis</i> var. <i>zymogenes</i>    | 2              | +          | -                    | +(1)<br>-(1)                    | +             | +       | -         | +                           | +             | +                |
| <i>Str. durans</i>                            | 7              | -          | -                    | -                               | -             | -       | -         | +                           | +             | +(4)<br>-(3)     |
| <i>Str. bovis</i>                             | 18             | -          | -                    | -                               | +(11)<br>-(7) | +       | +         | -                           | +             | -                |
| Unclassified                                  | 2              | -          | -                    | -                               | +             | -       | -         | +                           | +             | +                |
|   | 2              | -          | -                    | -                               | -             | +       | -         | +                           | +             | -                |
|   | 1              | -          | -                    | +                               | +             | +       | +         | +                           | +             | -                |
|   | 2              | -          | -                    | +                               | +             | +       | -         | -                           | +             | +                |
|   | 1              | -          | -                    | +                               | +             | +       | -         | -                           | +             | -                |
|   | 2              | -          | -                    | +                               | +             | +       | +         | -                           | +             | +                |
|   | 4              | -          | -                    | -                               | +             | +       | -         | -                           | +             | -                |
|   | 1              | -          | -                    | -                               | +             | +       | -         | -                           | -             | -                |
|   | 2              | -          | -                    | -                               | +             | +       | -         | -                           | +             | +                |
|   | 1              | -          | -                    | -                               | +             | -       | -         | -                           | +             | -                |
|   | 5              | -          | -                    | -                               | -             | +       | -         | -                           | +             | ±*               |
|   | 1              | -          | -                    | -                               | -             | -       | -         | -                           | +             | -                |
| Total   | 76             |            |                      |                                 |               |         |           |                             |               |                  |

\* ± = Slight growth.

Table 3 gives the cultural characters of seventy-six such strains which consistently failed to react with potent group D sera by the usual Lancefield technique but which all clearly precipitated when the group substance was concentrated (fraction A).

Whereas specific names can be assigned to fifty-two of these strains the remaining twenty-four, in accordance with opinions previously expressed (Shattock, 1945), are listed as unclassified. It is of interest that other strepto-

cocci with the cultural characters of the unclassified strains in Table 8, but which could be readily grouped without recourse to fraction A, have been frequently encountered. This lends further support to the serological assignment of such strains to group D.

Without a large-scale statistical survey it is not possible to assess the prevalence of group D strains with an apparent deficiency in group substance. The seventy-six strains described here were collected over a period of years and retained because they could not be grouped by the accepted serological

Table 4. *Numbers of group D strains from infant faeces reacting as fraction A only, compared with those reacting as crude HCl extracts*

| Species or variants                           | No. of strains tested | No. of strains reacting with group D sera |                    |
|---|-----------------------|---|--------------------|
|   |                       | As HCl extracts                           | As fraction A only |
| <i>Str. faecalis</i>                          | 126                   | 118                                       | 8                  |
| <i>Str. faecalis</i> var. <i>liquefaciens</i> | 46                    | 43  | 3                  |
| <i>Str. faecalis</i> var. <i>zymogenes</i>    | 24                    | 22  | 2                  |
| <i>Str. durans</i>                            | 3                     | 3   | 0                  |
| <i>Str. bovis</i>                             | 30                    | 20  | 10                 |
| Unclassified                                  | 81                    | 21  | 10                 |
| Total   | 260                   | 227                                       | 33                 |
| Percentage                                    |                       | 87.3                                      | 12.7               |

methods. Some indication of the incidence of such strains among group D streptococci from one source, infant faeces, is given in Table 4, for which I am indebted to Miss M. E. Sharpe of this laboratory. Of 260 strains 12.7% were grouped only by recourse to fraction A. There were no strains having biochemical properties resembling members of group D which could not be classified. Some further information on this point is furnished by Mattick & Shattock (1943). In an investigation on the numbers of group D streptococci occurring in English hard cheese it was found that of seventy-six cultures isolated from Cheddar cheese by a selective technique, and having the biochemical characters of group D streptococci, ten (13%) failed to precipitate with potent group D sera by the usual Lancefield technique. Four of the aberrant strains were kept and have since given definite reactions with group D sera, using fraction A as antigen.

## DISCUSSION

In a review of the streptococci, Sherman (1937) placed *Str. bovis* with *Str. salivarius*, *Str. equinus* and *Str. thermophilus* in what he termed the 'viridans group'. Schottmüller (1903) suggested *Str. viridans* as a name for streptococci giving zones of green discoloration on blood agar. This property, now known to be due to the production of hydrogen peroxide, is shared by many species of streptococci belonging to various serological groups. The application of the term 'viridans' to a collection which includes a proportion only of the



hydrogen peroxide forming species and also contains a species, *Str. thermophilus*, which is inert on blood agar, is unfortunate.

Biochemically *Str. salivarius* bears a marked resemblance to *Str. bovis*. However, the fermentation of starch and arabinose by *Str. bovis*, its higher maximum temperature for growth and its greater tolerance of bile serve to distinguish it from *Str. salivarius*. It is not proposed to discuss in detail the differentiation of these two species, as it has been fully described by Sherman (1987) and by Sherman, Niven & Smiley (1948). In addition to biochemical

Table 5. *Differentiation of species within group D*

| Species  | Haemolysis | Gelatin<br>lique-<br>faction | Strong<br>reduction<br>of<br>litmus<br>milk | Mannitol | Sucrose | Raffinose | Survival<br>at 60°<br>for<br>30 min. | Growth<br>at 45° | Growth<br>at<br>pH 9.6 |
|--|------------|------------------------------|---|----------|---------|-----------|--------------------------------------|------------------|------------------------|
| <i>Str. faecalis</i>                             | —          | —                            | +   | +        | ±       | —         | +                                    | +                | +                      |
| <i>Str. faecalis</i> var.<br><i>symogenes</i>    | +          | ±                            | ±   | +        | +       | —         | +                                    | +                | +                      |
| <i>Str. faecalis</i> var.<br><i>liquefaciens</i> | —          | +                            | +   | +        | +       | —         | +                                    | +                | +                      |
| <i>Str. durans</i>                               | ±          | —                            | —   | —        | —       | —         | +                                    | ±                | ±                      |
| <i>Str. bovis</i>                                | —          | —                            | —   | ±        | +       | +         | —                                    | +                | —                      |

± = varies from strain to strain.

studies they prepared type-specific sera for *Str. salivarius* but were unable to demonstrate any group relationship with group D. It is of interest to note that in this laboratory also no evidence of any serological affinity between *Str. salivarius* and group D has been obtained. Concentrated extracts prepared from strains of *Str. salivarius* gave no reactions with potent group D sera.

The preparation of specific group sera from typical strains of *Str. bovis* unequivocally places it with other intestinal streptococci in group D, though it is clearly a distinct species within the group, being distinguished from the other members by the tests shown in Table 5.

Mention must be made of two other species of streptococci which have been erroneously associated with group D.

*Str. uberis*, an inhabitant of the bovine vagina and occurring frequently in normal milk, is also associated with a form of bovine mastitis which is usually sporadic but which may assume epidemic proportions. *Str. uberis* has some of the cultural characteristics of group D streptococci, and Smith & Sherman (1942) have in fact included it in group D. Its serological grouping has, however, not yet been satisfactorily defined, although it is undoubtedly physiologically and antigenically related to group E (Little, 1989; Plastringe & Williams, 1989). The serological affinity of *Str. uberis* to members of group E has been confirmed in this laboratory by Jacob (1947). He investigated the physiological and antigenic characters of 128 strains of *Str. uberis* isolated by various workers and found that though they were closely related to group E there was no evidence of any relationship with group D. The group-specific substance of *Str. uberis* was not precipitated from HCl extracts with ethanol,

unlike group D streptococci, whilst Fuller's (1938) method of extraction gave better results than did Lancefield's extracts.

*Str. thermophilus* is another species which has yet to be grouped. Although its high maximum growth temperature of 47–50° may suggest that it is of intestinal origin there is no evidence in support of this. It is often found in milk and milk products, particularly those that have been pasteurized, but it has not been reported from human or animal sources. Abd-El-Malek & Gibson (1948), in a study of streptococci from pasteurized milk, grouped *Str. bovis* and *Str. thermophilus* together on biochemical grounds and suggested that they were closely allied. The sensitivity of *Str. thermophilus* to bile, its inability to ferment a large number of carbohydrates, and its characteristic pleomorphic morphology clearly distinguish it from *Str. bovis*. As yet *Str. thermophilus* has not been grouped serologically, although typing sera have been prepared in this and other laboratories (Zollikofer & Janaik, 1944). One fact has been established in this laboratory: it bears no antigenic relationship to group D nor does it precipitate with sera of any of the groups A to N.

It is appropriate to discuss briefly the present position of group D streptococci in relation to each other, as some confusion of nomenclature is still apparent in recent literature. Evans & Chinn (1947), in a paper on the 'Enterococci with special reference to their association with human disease', divided their collection of group D streptococci on the basis of cultural tests, susceptibility to two strains of phage, and agglutination tests apparently carried out with unabsorbed sera. They found that the type of haemolysis and liquefaction were unrelated to other characteristics and suggest that their main group of seventeen strains, comprising cultures having the properties of *Str. faecalis*, *Str. faecalis* var. *liquefaciens* and *Str. faecalis* var. *zymogenes*, should all be included under the name *Str. zymogenes* as having prior claim to specific name. *Str. zymogenes* was originally described as *Micrococcus zymogenes* by MacCallum & Hastings (1899), but the study of colonial appearance on blood agar was at that time yet to be introduced, and their admirable description might equally well have applied to *Str. faecalis* var. *liquefaciens*, identified by Orla-Jensen (1919) with the *Micrococcus casei amari* of Freudenreich. It is of interest to note in this connexion that non-haemolytic proteolytic strains have been associated with pathological conditions (e.g. Elser & Thomas, 1936). The specific name *Streptococcus faecalis*, introduced and clearly defined by Andrewes & Horder (1906), has been accepted and its description extended by recognized authorities in more than one field (e.g. Dible, 1921; Sherman, 1938). To discard this well-established name at this stage would merely cause confusion where some order has been established. Although the close relationship of *Str. faecalis* to the proteolytic and haemolytic enterococci is fully appreciated (Sherman, Stark & Mauer, 1937), and although it is recognized (Shattock & Mattick, 1943; Elser & Thomas, 1936; Sharpe, 1948) that haemolysis, or even proteolysis, is not fundamentally associated with antigenic pattern, no useful purpose would be served at present by discarding the names *liquefaciens* and *zymogenes* as varieties of the central type. Information on the type serology of group D is insufficient for practical use at present, but doubtless it is only a question of

time before a serological division similar to that worked out for group A will enable significant and fundamental distinctions within group D to be made.

It is acknowledged (Shattock, 1945), and has been demonstrated again in this paper, that there are no clear-cut lines of demarcation between the various species in group D, but certain well-defined members may be identified and *Str. bovis* has now been added to their ranks (Table 5). If specific names are to retain any significance, aberrant strains should be described as belonging to the serological group and their cultural characters recorded until more precise (e.g. serological typing) information becomes available. In this connexion, particularly where growth under certain specified conditions is observed (e.g. Shattock & Hirsch, 1947), the necessity of adhering strictly to a uniform technique cannot be emphasized too strongly.

*Group D specific substance.* The concentration of the group-specific substance by precipitation from HCl extracts with ethanol not only gives a method of grouping refractory strains but also raises the question of the nature of the specific substance of group D. It has been assumed, apparently by analogy with groups A, B and C (Lancefield, 1940-1; Wilson & Miles, 1946), that the group-specific substances of the *Streptococcus* groups A-N are all complex carbohydrates. Preliminary work in this laboratory based on crude fractionation and colour tests, however, indicates that the group-specific substance of D is probably protein. This agrees in part with the work of Foley & Wheeler (1945), who, as already noted, found that in some group D strains the group-specific substance was protein. Their finding of a carbohydrate group-specific substance in another strain, however, is not easy to understand. That there should be within the same group so-called group-specific substances of totally different chemical nature seems to conflict with the criterion on which the grouping of the streptococci is based. Whether the substance be protein or carbohydrate is immaterial, but the use of the term 'group-specific' to a bacterial component should imply that it is common to all members of the group. More precise information on the chemical nature of the group-specific fraction of group D would doubtless help to explain the unusual difficulties encountered in serological studies on this group.

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## Observations upon the Cytology of Corynebacteria and Mycobacteria

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**SUMMARY:** Bacilli in young cultures of *Corynebacteria* and *Mycobacteria* are multicellular. The individual cells are almost spherical, and a single bacillus may contain from one to twelve or more units. Reproduction may take place by division of the constituent cells, followed by simple fission of the bacillus, or alternatively by fragmentation into single cells which subdivide without separating, and grow into multicellular bacilli once more. The latter phenomenon may account for previous descriptions of life cycles in these genera.

The nuclear units are small, spherical granules, resembling those of some species of cocci. The characteristic morphology of *Corynebacteria* and *Mycobacteria* is an artefact, resulting from drying and heat-fixation. These genera do not appear to possess any morphological characters in common with the true *Actinomyces*, with which they are at present classified.

In the study of the morphology of the *Corynebacteria* great confusion has been caused by attempts to interpret the appearance of *Corynebacterium diphtheriae* when stained by the various diagnostic methods which have been employed in routine work. It is not proposed to attempt to review the literature on the nature of the metachromatic granules which has accumulated in the last 50 years, most of which ignores the fallacy of attempting to base cytological conclusions upon the evidence of heat-fixed material. Various members of the group have also been described as 'barred' in appearance, and it is the opinion of the author that these two characteristics have often been confused. In the *Mycobacteria* also, both granules and barring have often been described. Porter & Yegian (1945) have shown that the granules in *Mycobacterium tuberculosis* are staining artefacts, and have also demonstrated chromatinic bodies in this organism, by Robinow's method. Brieger & Robinow (1947) have confirmed this latter observation in the avian type of bacillus. Discrete Feulgen-positive granules have also been observed in *M. tuberculosis* by Epstein, Ravich-Birger & Svinkina (1936). Brieger & Robinow (1947) tried to demonstrate the transverse septa which consideration of its morphology led them to expect in this organism, but failed to do so. In the present paper it is intended to show that these septa do in fact exist. They also appear quite clearly in an electron micrograph by Brieger, Crowe & Cosslett (1947), although their presence is not commented upon in the text. The similar multicellular structure of *C. diphtheriae* is demonstrated by Burdon (1946) in drawings intended to illustrate the lipid content of the organism.

**Materials and methods.** The bacteria employed were freshly isolated strains wherever possible, and were grown upon routine culture media. Preparations were stained by Robinow's (1945) methods for nuclear materials and cell walls. Wet preparations were used throughout.

## Observations

A large number of preparations were made from eight strains of *C. diphtheriae* (four *gravis*, two *mitis*, two *intermedius*) isolated from routine throat swabs. When stained by Neisser's stain these showed the characteristic morphology of the species, with obvious metachromatic granules (Pl. 1, fig. 1). When stained by tannic-acid-violet, they appeared much larger and were seen to consist of from one to six or seven almost-spherical cells (Pl. 1, fig. 2). Acid-Giemsa preparations showed that each cell was occupied by strongly

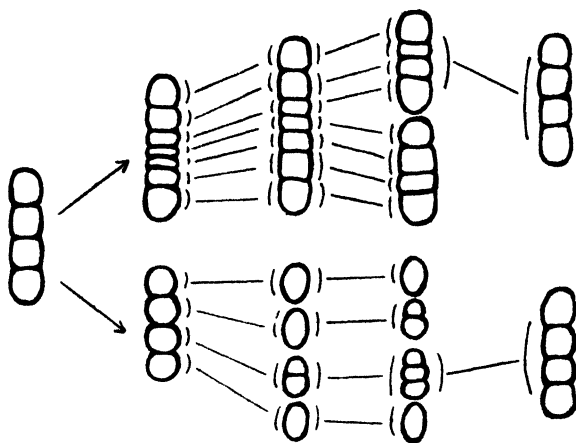


Fig. 1

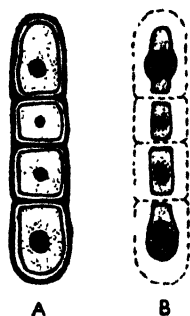


Fig. 2

Fig. 1. *Mycobacterium* or *Corynebacterium*. Alternative modes of reproduction. Above. Division of bacillus preceded by multiple cell division. Below. Fragmentation and regeneration from individual cells.

Fig. 2. *Corynebacterium diphtheriae*. A. Interpretation of actual morphology. B. 'Typical' morphology as produced in fixed, dried preparations. Note the effect of shrinkage.

stained protoplasm containing a small central granule (Pl. 1, figs. 3, 4). Other *Corynebacteria* of human origin, which did not exhibit metachromatic granules when stained by Neisser's stain, showed the same cytological appearances (Pl. 1, figs. 5, 6). These included two strains from the nasal passage and two from the vagina. The granule divided before the cell, and its appearance was very similar to that of the nuclear granules which have been reported in some cocci (Knaysi, 1942; Bisset, 1948). These appearances were best seen in young cultures; after 18 to 24 hr. growth they became difficult to stain.

Two methods of reproduction appeared to occur (Fig. 1). Division of the bacillus was sometimes preceded by cell division, producing a stage with a large number of small cells which then divided into two normal bacilli. Alternatively the bacillus was sometimes observed in the process of fragmentation into its constituent cells, from which, by growth and cell division, the bacilli were reformed. These two methods of reproduction occurred simultaneously in a single culture.

The *Mycobacteria* which were examined were stock cultures of *M. tuberculosis*

(cold-blooded) and *M. phlei*, and one newly isolated strain of *M. tuberculosis* (human). All were difficult to stain; but a preliminary treatment with warm 70 % ethanol rendered them more readily stainable. The resemblance of their cytological structure to that of the *Corynebacteria* was very great (Pl. 1, figs. 7-10). The tubercle bacilli also consisted of one to twelve or more small cells, each containing a chromatinic granule, although the latter, because of its small size, was difficult to resolve.

## DISCUSSION

The 'characteristic morphology' of *C. diphtheriae* appears to be an artefact. The barred appearance of this species, and also of the tubercle bacillus, in dried, heat-fixed films, is due to shrinkage of the individual cell-contents away from the cell wall, producing gaps between them (Fig. 2). The metachromatic granules are probably mere condensations of stainable material within the dried cell. The present study has not attempted to add to the already considerable mass of contradictory evidence upon the nature of the material of which these metachromatic granules are composed. They do not correspond to the nuclear granules, as these are present in other types of *Corynebacteria* which do not exhibit metachromatic staining. It is probable, however, that the metachromatic material accumulates around the nuclear granules, as indicated in the diagram.

Some of the life-cycle theories which have been suggested in the tubercle bacillus may well be based upon observations of a system of reproduction involving fragmentation of the bacilli into their constituent cells. This multicellular structure has also a bearing upon reports of true branching in these genera. None of the strains examined possessed a cytological structure resembling that of the sporing *Actinomyces* as described by Klieneberger-Nobel (1947), in which the individual cell is branched. Nor do the branching tubercle bacilli illustrated by Brieger & Robinow (1947) appear to possess such a structure. There seems little morphological basis for the belief that the 'higher bacteria' are related to the true *Actinomyces*.

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## EXPLANATION OF PLATE

- Fig. 1. *C. diphtheriae* (*gravis*), Neisser's stain.  $\times 3000$ .
- Fig. 2. *C. diphtheriae* (*gravis*), tannic-acid-violet.  $\times 3000$ .
- Fig. 3. *C. diphtheriae* (*gravis*), acid-Giemsa.  $\times 3000$ .
- Fig. 4. *C. diphtheriae* (*intermedius*), acid-Giemsa.  $\times 3000$ .
- Fig. 5. *Corynebacterium* sp. (human vaginal origin), tannic-acid-violet.  $\times 3000$ .
- Fig. 6. *Corynebacterium* sp. (human vaginal origin), acid-Giemsa.  $\times 3000$ .
- Fig. 7. *M. tuberculosis* (human), acid-Giemsa.  $\times 3000$ .
- Fig. 8. *M. tuberculosis* (cold-blooded), acid-Giemsa.  $\times 3000$ .
- Fig. 9. *M. tuberculosis* (cold-blooded), tannic-acid-violet.  $\times 3000$ .
- Fig. 10. *M. phlei*, tannic-acid-violet.  $\times 3000$ .

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ADDENDUM (17 November 1948). In the 1948 edition of Bergey's *Manual of Determinative Bacteriology* (6th ed. Baltimore: Williams and Wilkins) the *Corynebacteria* are separated from the *Mycobacteria* and placed with the *Eubacteria*. The cytological findings in the present paper are even more at variance with this classification than with that in the 1939 edition.



Figs. 1-10



# The Serological Comparison of Strains of Influenza Virus

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**SUMMARY:** The antigens of eight strains of influenza virus were compared by a simple and economical complement-fixation technique in which drops on Perspex sheets replaced larger volumes of reagents in racks of tubes. By this means more extensive tests were made with a limited supply of material than by standard tube techniques.

The specific antisera were prepared in mice, thus avoiding the elaborate isolation precautions necessary for ferrets, and sera from large groups were pooled to minimize individual variations between animals. The antigens were standardized in terms of the amount of complement fixed in the presence of excess homologous antiserum. The comparison of the strains is presented in the form of index numbers, ranging from 0 to 1.0; 1.0 represents complete identity of two strains, while the smaller numbers are taken to represent the degree of antigenic relationship between strains. Of the six strains examined which were isolated in different years, all were serologically distinct though the two B strains were closely similar and two of the A strains were fairly closely related. On the other hand, the three strains of influenza A virus isolated in 1947, two in London and one in the U.S.A., were identical.

Knowledge of the antigenic differences between strains of influenza virus is important both in the study of the epidemiology of influenza and in the choice of the best components for a vaccine. Most comparisons have been made in one of three ways. The first method, using the chick red cell agglutination phenomenon (Hirst, 1943), has the advantage of an apparent simplicity. This is apt to be somewhat misleading, for not only do different batches of red cells vary in behaviour (Stuart-Harris, 1943), but variations in avidity of the antigen must also be taken into account. Furthermore, haemagglutination by some freshly isolated strains of virus is inhibited by normal serum (Francis, Salk & Brace, 1946). To crown all these difficulties any 'reasonable' answers can only be obtained by assuming a law of reciprocal relations between any two virus strains.

The other methods of comparison are by cross-immunization or by the serum neutralization test in mice (Francis & Magill, 1938; Magill & Francis, 1938; Smith & Andrewes, 1938). The possible fallacy in these methods is that all the strains need to be mouse adapted, and Hirst (1947) has recently shown that adaption to mice entails the replacement of the strain growing in eggs by another variant which may be antigenically different from the 'egg' virus. Moreover, persistent failure has met all attempts to adapt certain strains of influenza B virus to mice.

The complement-fixation test is more difficult to perform, but this added labour is in our opinion justified by the superiority of the results obtained. This paper describes a method of conducting the test so that strains of influenza virus may be compared with precision.

## MATERIAL AND METHODS

For an adequate titration it is essential to be able to vary both the serum dilutions and the antigen dilutions in a 'chess-board' experiment. Such tests demand a large number of tubes as well as considerable volumes of reagents. When either of the reagents is anticomplementary in the zones in which the titration must be conducted, it becomes also necessary to vary the complement, and it is impossible to make such a test without splitting every experiment into a number of orientating approaches so that the final test is a selection of the variables designed to give the maximum information with a reasonable number of tubes. In order to be able to carry out spaciouly conceived tests without this wearisome preliminary orientation, we have designed a complement-fixation test so that the reaction is carried out not in tubes, but in drops on Perspex sheets.

*Apparatus*

*Perspex sheets.* The Perspex sheets were 16 in. square and  $\frac{1}{8}$  in. thick. The top surface of the sheet was engraved with a centrally placed grid of 144 in. squares in 12 rows of 12. The rows of squares were numbered along the left-hand margin 1 to 12 from top to bottom, and the columns were numbered along the top margin from left to right *a* to *n*, omitting *i* and *l*. Twelve such plates were made, and each was marked with a letter *A* to *L* inclusive (Pl. 1, fig. 1).

*Rack.* A rack was constructed to hold these twelve Perspex sheets in the horizontal position. It was made of laminated bakelite and brass tubing, stood on brass legs 2 in. long and had a carrying handle at the top. The Perspex sheets could be slid on to their narrow supporting shelves and were held  $\frac{1}{8}$  in. apart. The sheets were held in position by inserting a metal rod (Pl. 1, fig. 2).

*Boxes.* Two identical boxes were made of sheet brass to accommodate this rack. They were constructed with a top flange covered with rubber so that by screwing down the lid they could be made airtight. The boxes were kept saturated with water vapour by putting a layer of absorbent cotton-wool in the bottom and keeping this moist with distilled water. One of the boxes was held in a refrigerator at 4°, and the other in a hot-room at 87°.

*Dropping pipettes.* Dropping pipettes were made to deliver 20 mm.<sup>3</sup> of water (Morse gauge 57) and 40 mm.<sup>3</sup> of water (Morse gauge 48). The delivery of these volumes was found to be sufficiently accurate over the whole range of reagent dilutions used, irrespective of their varying surface tensions.

*Reagents*

*Strains.* The following strains were examined.

Influenza A: PR8, WS (both mouse adapted); Barratt (BAR), Williams (WIL)—isolated London, 1947; Rhodes (RHO)—isolated U.S.A., 1947.

Influenza B: Lee (mouse adapted); Crawley (CRA)—isolated London 1946.

Swine influenza: Shope 15 (mouse adapted).

The strains were stored as infected allantoic fluids mixed with an equal volume of 10 % horse-serum broth and kept in sealed glass ampoules at -76°.

It is necessary to emphasize that at the present time many of the classical

influenza strains have had so varied a history that it cannot be assumed, for instance, that the strain called PR8 in this laboratory is now identical with PR8 strains used in laboratories in other parts of the world.

*Antigens.* Allantoic fluids infected with influenza virus contain two complement-fixing antigens (Friedewald, 1948; Wiener, Henle & Henle, 1946). One antigen is associated with the elementary body and is strain specific, the other is the soluble antigen which is group specific. For our purpose the elementary body antigen alone is required. The stored, infected, allantoic fluids were diluted  $10^{-2}$  in 0.85 % saline and passed intra-allantoically to 10-day chick embryos. After 48 hr. at  $35^{\circ}$  the virus was harvested. The elementary bodies were partially purified and concentrated five-fold by absorption and elution from red cells by the method of Francis & Salk (1942). The Shope strain could not be satisfactorily eluted (Burnet, Beveridge, McEwin & Boake, 1945), and so with this strain the infected allantoic fluids were harvested bloodlessly and the elementary bodies partially purified and concentrated by high-speed centrifugation.

The eluted elementary bodies in physiological saline were tested for haemagglutination by the Salk pattern test (Salk, 1944), using a final concentration of chick cells of 0.25 %. The titres ranged from 1/1000 to 1/4000. The antigens were stored in sealed glass ampoules at  $-76^{\circ}$ .

*Antisera.* Immune mouse sera were used, the mice being infected with allantoic fluid given intranasally. In the past it has been usual to use immune ferret sera in the antigenic analysis of strains of influenza virus, but mouse sera possess several advantages. As a pool from at least forty mice is used, individual variations are minimized, and further, as cross-infection cannot be demonstrated among mice kept in different cages (Laidlaw, Smith, Andrewes & Dunkin, 1935) it is possible to prepare sera from a number of different strains without adopting the strict isolation procedures which would be necessary with ferrets. In addition, mouse sera contain no natural haemolysins for sheep cells.

The infected allantoic fluids of the mouse-adapted strains were diluted so that they would cause very few deaths but would produce some consolidation of the lungs. Usually such fluids were diluted  $10^{-5}$  in saline, and 0.05 ml. was given intranasally.

With the unadapted A strains the infected allantoic fluid was passaged undiluted. Although there was no obvious change in the lungs of the mice, potent immune sera were obtained. That multiplication of the virus does in fact take place has been demonstrated by Hirst (1947), and is also shown by the fact that there is no antigenic response if the virus in the allantoic fluid is first killed with formalin.

With the unadapted B strain (CRA) undiluted allantoic fluid killed most of the mice and so the fluid was given diluted  $10^{-2}$ .

After 18 days the mice were bled from the heart, the pooled blood was allowed to clot and then centrifuged at 18,000 r.p.m. for 15 min. The clear serum was dispensed into glass ampoules which were sealed and stored at  $-76^{\circ}$ . Since mouse serum lacks the  $C^3$  component of complement

(Brown, 1948), it might be supposed that inactivation would be unnecessary. In fact, unheated mouse serum was strongly anticomplementary and, moreover, fixed more complement in the presence of influenza antigens. To avoid these non-specific fixations the sera must be heated. Casals & Palacios (1941) have shown in the titration of other antibodies in mouse sera that the optimum inactivation temperature is 60°. However, at this temperature, the influenza antibodies are rapidly destroyed. Throughout this work the mouse sera were inactivated immediately before use for 30 min. at 55°.

*Complement.* The complement was pooled guinea-pig serum stored in sealed glass ampoules at -76°.

*Diluent.* The diluent was based on the data of Mayer, Osler, Bier & Heidelberger (1946). Stock: NaCl 85.0 g.; 5,5-diethylbarbituric acid 5.75 g.; sodium-5,5-diethylbarbiturate 3.75 g. Dissolve the acid in 500 ml. of hot glass-distilled water, add the other components and make up to 2000 ml.; then add  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  1.68 g. and  $\text{CaCl}_2$  0.28 g. Autoclave at 15 lb./sq.in. for 20 min., pH = 7.2. Store at 4°. For use the stock is diluted 1/5 in glass-distilled water.

*Sensitized sheep erythrocytes.* Sheep blood was stored in Alsever's solution and used for periods up to 4 months (Bukantz, Rein & Kent, 1946). Immediately before use the cells were washed three times, diluted to approximately 0.2% and then accurately standardized in a photoelectric densitometer. The cells were maximally sensitized by the method of Wadsworth, Maltaner & Maltaner (1938), which, using Burroughs Wellcome haemolysin, entailed adding to the cell suspension an equal volume of haemolysin diluted 1/400. Sensitization was allowed to proceed for 1 hr. at 37°.

### *Conduct of the test*

To illustrate the method a titration of a PR8 immune mouse serum is presented in detail. In test-tubes a series of eleven twofold dilutions of the serum are made, from 1/1 to 1/1024, and sixteen dilutions of complement as in Table 1. To provide enough of these dilutions for twelve Perspex sheets, only 1.7 ml. of guinea-pig serum is required. The antigen dilution is also prepared, in this case so as to contain 1000 haemagglutination units.

A Perspex sheet (say B) is laid on the bench. Using a 20 mm.<sup>3</sup> dropping pipette the serum dilutions are first distributed. Row 12 receives one drop of diluent (control row), row 11 receives one drop of serum dilution 1/1024, row 10 receives one drop of serum dilution 1/512, and so on. Then to each square is added a drop of the appropriate complement dilution according to the scheme in Table 1. Finally, a drop of diluent is added to the pool in each square, as B is the serum control sheet. No deliberate mixing of the drops is necessary. Another Perspex sheet (say C) is similarly prepared except that the third drop of each pool is the antigen.

Each sheet takes about 12 min. to set out, and as it is completed it is put away in the rack which is already inside the box in the cold room. When the last plate has been inserted the top of the box is screwed down and left to stand overnight. Next day the rack is removed from the cold room. To each square

on the Perspex sheets 40 mm.<sup>3</sup> of sensitized sheep cells are added with a dropping pipette. Again no mixing is required. The sheets in the rack are now transferred to the water-saturated box in the 37° hot room, and the lid screwed down. At the end of 2 hr. the rack is removed from the box and allowed to stand at room temperature for half an hour or so, to allow the surface film of moisture to evaporate from the sheets.

Table 1. *Complement dilution table, with scheme for the distribution of the complement dilutions on Perspex sheets*

| Tube no. | Dilution 1 in | mm. <sup>3</sup> * | log <sub>10</sub> mm. <sup>3</sup> | Distribution on Perspex sheets |          |          |
|----------|---------------|--------------------|------------------------------------|--------------------------------|----------|----------|
|          |               |                    |                                    | Columns                        |          |          |
|          |               |                    |                                    | Row 1-3                        | Row 4-7  | Row 8-12 |
| 1        | 3·2           | 6·3                | 0·8                                | <i>a</i>                       | —        | —        |
| 2        | 5             | 4·0                | 0·6                                | <i>b</i>                       | —        | —        |
| 3        | 8             | 2·5                | 0·4                                | <i>c</i>                       | <i>a</i> | —        |
| 4        | 12·6          | 1·6                | 0·2                                | <i>d</i>                       | <i>b</i> | —        |
| 5        | 20            | 1·0                | 0·0                                | <i>e</i>                       | <i>c</i> | <i>a</i> |
| 6        | 32            | 0·63               | 1·8                                | <i>f</i>                       | <i>d</i> | <i>b</i> |
| 7        | 50            | 0·4                | 1·6                                | <i>g</i>                       | <i>e</i> | <i>c</i> |
| 8        | 80            | 0·25               | 1·4                                | <i>h</i>                       | <i>f</i> | <i>d</i> |
| 9        | 100           | 0·2                | 1·3                                | —                              | —        | <i>e</i> |
| 10       | 126           | 0·16               | 1·2                                | <i>j</i>                       | <i>g</i> | <i>f</i> |
| 11       | 159           | 0·13               | 1·1                                | —                              | —        | <i>g</i> |
| 12       | 200           | 0·10               | 1·0                                | <i>k</i>                       | <i>h</i> | <i>h</i> |
| 13       | 250           | 0·08               | 2·9                                | —                              | <i>j</i> | <i>j</i> |
| 14       | 316           | 0·06               | 2·8                                | <i>m</i>                       | <i>k</i> | <i>k</i> |
| 15       | 400           | 0·05               | 2·7                                | —                              | <i>m</i> | <i>m</i> |
| 16       | 500           | 0·04               | 2·6                                | <i>n</i>                       | <i>n</i> | <i>n</i> |

\* Equivalent volume of undiluted complement in 20 mm.<sup>3</sup> drop.

The test is read by placing the Perspex sheets flat on a white background with a strong light overhead. Where the cells have not been haemolysed they collect in a central dot (Pl. 1, fig. 3), rather as the cells in the control tube collect in a button at the bottom of the tube in the Salk test.

By observing the density of this dot, the end-point is taken as that square which by inspection shows half the cells haemolyzed. With the complement intervals chosen this point is usually sharp and easy to assess. Frequently one square shows complete haemolysis and the next no haemolysis, in which case the end-point is taken as the geometric mean of the amounts of complement in the two squares.

The data are given in Table 2. On the serum control sheet, B, the square for 50 % haemolysis is noted for each serum dilution. From this the amount of complement required is obtained by reference to Table 1. In the same way on sheet C the amounts of complement required for 50 % haemolysis in the presence of antigen are noted. By subtracting the first value from the second, the amount of complement fixed in the antigen-antibody reaction is obtained. The unit is taken as the minimal amount of complement needed to produce 50 % haemolysis in the presence of the antigen, in this case 0·05 mm.<sup>3</sup> Dividing the mm.<sup>3</sup> of complement fixed by 0·05, we obtain the number of units fixed



at each serum dilution, here expressed as the logarithm. In Fig. 1 (solid line) the log units fixed have been plotted against log serum-dilutions. Parallel tests have shown that the fixation on the sheets is identical with that occurring in tubes using the same reagents but with 200 mm.<sup>3</sup> volumes in the tubes instead of 20 mm.<sup>3</sup> drops. In Fig. 1 and in subsequent tests the undiluted serum is

Table 2. Complement fixation of PR 8 immune serum with 1000 haemagglutinin units of homologous antigen. Calculation of the log-units fixed

| Row | Serum dilution one in | B, serum control mm. <sup>3</sup> C (x) | C, antigen 1000 units  |       |                             |                                 |
|-----|-----------------------|---|------------------------|-------|-----------------------------|---------------------------------|
|     |                       |   | mm. <sup>3</sup> C (y) | (y-x) | log <sub>10</sub> (y-x) (z) | log <sub>10</sub> units (z-2.7) |
|     |                       |   |                        |       |                             |                                 |
| 1   | 1                     | 1.0                                     | 4.0                    | 3.0   | 0.48                        | 1.78                            |
| 2   | 2                     | 0.4                                     | 3.2                    | 2.8   | 0.45                        | 1.75                            |
| 3   | 4                     | 0.25                                    | 2.5                    | 2.25  | 0.35                        | 1.65                            |
| 4   | 8                     | 0.16                                    | 2.0                    | 1.84  | 0.26                        | 1.56                            |
| 5   | 16                    | 0.10                                    | 1.3                    | 1.20  | 0.08                        | 1.88                            |
| 6   | 32                    | 0.08                                    | 0.63                   | 0.55  | 1.74                        | 1.04                            |
| 7   | 64                    | 0.07                                    | 0.25                   | 0.18  | 1.25                        | 0.55                            |
| 8   | 128                   | 0.06                                    | 0.06                   | 0     | —                           | —                               |
| 9   | 256                   | 0.06                                    | 0.05                   | -0.01 | —                           | —                               |
| 10  | 512                   | 0.06                                    | 0.05                   | -0.01 | —                           | —                               |
| 11  | 1024                  | 0.06                                    | 0.05                   | -0.01 | —                           | —                               |
| 12  | —                     | 0.06                                    | 0.05*                  | -0.01 | —                           | —                               |

C=equivalent volume of undiluted complement.

\* 1 unit=0.05 mm.<sup>3</sup> complement.

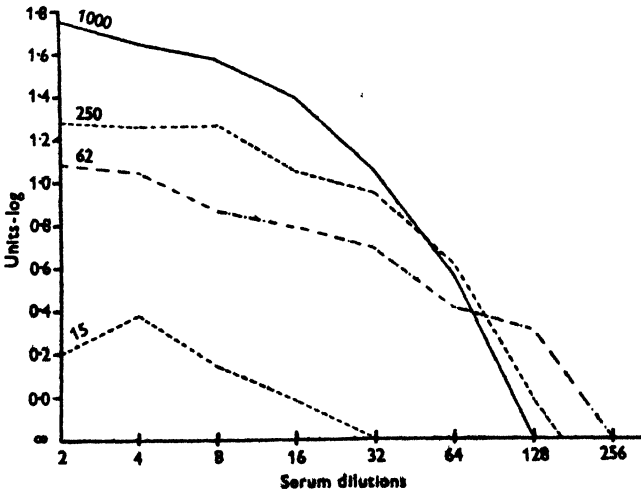


Fig. 1. PR 8 immune serum titrated with four different concentrations of homologous antigen of 1000, 250, 62 and 15 haemagglutinin units.

omitted, for with such strong reagents the number of units fixed in repeated tests is variable; and also, as will be demonstrated, the antibodies in the 1/2 dilution are so greatly in excess of the amounts of antigen available, that no information is gained. By this omission a great saving of serum was effected.

*Miscellaneous precautions.* It has been found convenient, when setting out the drops on the Perspex, to use a sheet of brass as a backing plate. This increases the internal reflexions and makes it easier to see how many drops have been placed on a square. At a later stage when the sensitized cells are being added it is preferable to use a backing plate of black bakelite. For reading the test a sheet of white erenoid has been found useful. The exact height from which the drops are delivered on to the Perspex sheet varies slightly with the prevailing humidity. Usually a height of 2–3 cm. is convenient. Occasionally the drops tend to ricochet on one another, but this tendency can be countered by slightly increasing the dropping height. Each sheet is completed and put away in the cold before the next is commenced. This may not be necessary when very few sheets are being set up, but in our experience of large series too much evaporation occurs if the sheets are left in the laboratory during the whole procedure. Even in the water-saturated box at 4°, a certain amount of evaporation does occur overnight, but this is not sufficiently marked to affect the complement, although no doubt if it could be entirely avoided the complement titre would be slightly higher. It has been found that even when the reaction is carried out in the usual manner in test-tubes with fixation for 18 hr. in the cold, a slightly higher complement titre is obtained by preventing surface evaporation by a layer of liquid paraffin in each tube.

In the design of the rack the space between the individual sheets is  $\frac{1}{8}$  in. This distance should not be decreased very much as, when the plates are too close, there is a tendency for the water in the drops to distil over on to the under-side of the sheet immediately above. It was found that the top sheet in our rack showed a greater degree of evaporation than the other sheets because of the close proximity of the bakelite top of the rack which cooled at a different rate. This was avoided by keeping sheet A always in position in the rack but not using it in the test. Obviously if the apparatus were redesigned the rack could be suitably modified.

The reading of the test depends, as has been described, on the fact that the unhaemolyzed cells collect in a dot in the centre of the pool. This is presumed to occur because the haemolysin dilution for maximal sensitization is close to the agglutination titre of the haemolysin serum. The effect is enhanced by sensitizing the cells for a full hour before use. Occasionally it will be found that on first reading the cells are not sharply confined to a central dot. In such cases a slight rotary twist of the sheet will clarify the pattern.

After reading, the sheets are placed in a large photographic dish and covered with water containing 0.2 % HCl. After  $\frac{1}{2}$  hr. or so, they are washed in running cold water, using a swab of absorbent cotton-wool to clean the surface, rinsed in distilled water and placed in the rack which is laid on its side so that the sheets drain vertically. They are left at room temperature to dry overnight and are then stored in a dust-free box. It is important not to rub the dry sheets with a cloth because Perspex easily becomes electrically charged, and it is impossible to deliver drops on to such a sheet; the drops are dragged off the end of the pipette by electrostatic attraction before they have grown to the size expected under the pull of gravity alone.

*The standardization of the antigen*

A PR8 immune mouse serum was titrated in the manner described, using four different antigen concentrations, measured by haemagglutination, of 1000, 250, 62 and 15 haemagglutinin units (Fig. 1). It will be seen that the antigen concentration determines the maximal amount of complement which can be fixed, because in the stronger concentrations of serum the antibodies are greatly in excess of the binding capacity of even the strongest antigen used. The maximal number of units fixed, is, therefore, independent of the antibody level over a wide range, as is shown by the titration of mouse sera of high and low titres with 250 haemagglutinin units of homologous antigen (Fig. 2).

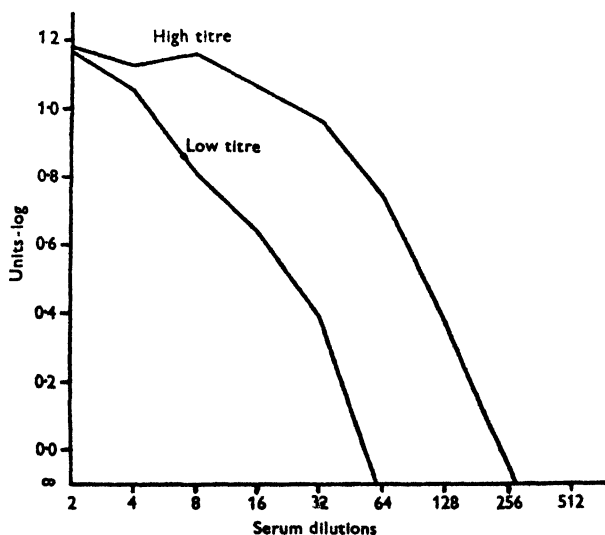


Fig. 2. Titration of two PR8 immune sera, one of low and one of high titre, with homologous antigen at a concentration of 250 haemagglutinin units.

This observation suggested a simple method of adjusting all the antigens to a standard concentration. For the main test the antigen is used at a constant concentration, arbitrarily chosen so that, in the presence of  $1/2$  homologous mouse antiserum, the amount of complement maximally fixed shall be 1.0 mm.<sup>3</sup> (about 1.2 log units).

Each antigen is standardized against its homologous antiserum in the following way. Complement dilutions (1–8, Table 1) are prepared in tubes and also antigen dilutions, usually from  $1/2$  to  $1/32$ . Sufficient inactivated homologous mouse serum is diluted  $1/2$ . Drops of the serum dilution are distributed along six rows (1–6) for the first eight columns. Complement is then added to the columns corresponding with the tube number in Table 1. Finally, to row 6 a drop of the  $1/32$  antigen is added, to row 5 a drop of the  $1/16$  antigen and so on, the first row acting as the serum control.

The calculation for PR8 is shown in Table 8, and the curve is drawn in Fig. 8 with a number of other antigen titrations. In this case the ordinates

are not log units but  $\text{mm}^3$  of complement fixed expressed as the logarithm. From this figure it is easy to determine the antigen concentration required to give a fixation of  $1.0 \text{ mm}^3$  (0.0).

Table 3. Example of the standardization of a PR8 antigen, with PR8 Immune Serum 1/2

| Row | Antigen dilution 1 in | $\text{mm}^3 C$<br>(y) | (y-0.5)<br>(z) | $\text{Log}_{10} z$ |
|-----|-----------------------|------------------------|----------------|---------------------|
| 1   | --                    | 0.5                    | —              | —                   |
| 2   | 2                     | 2.0                    | 1.5            | 0.18                |
| 3   | 4                     | 1.5                    | 1.0            | 0.00                |
| 4   | 8                     | 1.0                    | 0.5            | 1.70                |
| 5   | 16                    | 0.8                    | 0.3            | 1.48                |
| 6   | 32                    | 0.63                   | 0.13           | 1.11                |

$C$  = equivalent volume of undiluted complement.

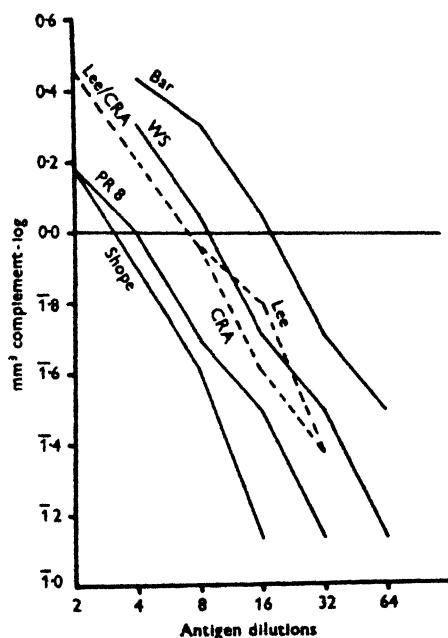


Fig. 3. The standardization of six antigens. See Table 3.

With any one strain it has been found that once the antigen level has been fixed in relation to the haemagglutinin titre, the latter can be used to determine the correct concentration. But the relation between the antigen level and the haemagglutinin level is not the same with every strain (Table 4). We are forced to conclude that the number of particles causing a given standard degree of haemagglutination is not identical in every strain.

#### Fixation with normal mouse sera

Antigens of the standard concentration fix a small amount of complement with normal mouse sera, especially in the stronger serum concentrations. This is illustrated in Fig. 4, where a normal mouse serum is titrated first without antigen (solid line) and then with antigen (dotted line). The ordinates here are

the logarithms of the mm.<sup>3</sup> of complement fixed. It will be seen that over a considerable range the curve of fixation with antigen is shifted up 0.2 unit, and this has been found to be true with all the antigens used. On occasion the

Table 4. *Relation between standard antigen dilution and haemagglutinin titre*

| Strain  | Haemagglutinin<br>in titre | Dilution for<br>standard antigen |
|---------|----------------------------|----------------------------------|
| PR 8    | 2000                       | 4                                |
| BAR     | 4000                       | 16                               |
| WS      | 1000                       | 8                                |
| Shope   | 1000                       | 4                                |
| Lee     | 4000                       | 8                                |
| Crawley | 4000                       | 8                                |

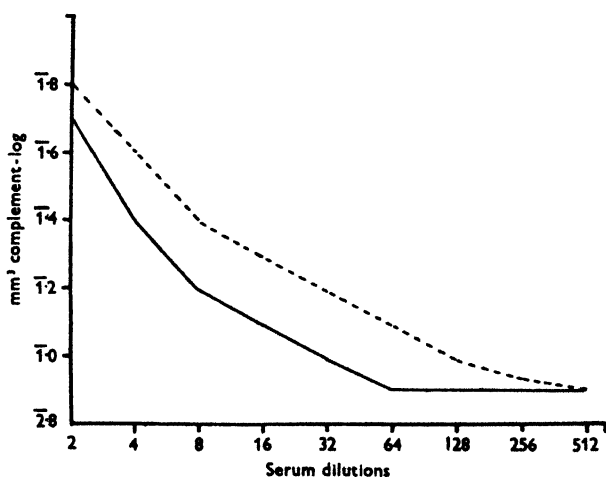


Fig. 4. Fixation of complement by normal mouse serum with standard antigen (dotted line) and without antigen (solid line).

shift is less than this, but it is never more. In any serum titration, therefore, the non-significant fixation is calculated in the manner shown in Table 5. To each value of complement fixed in the serum control, expressed as the logarithm, is added 0.2. These new values are then treated in the way already described and the log units fixed are calculated. It frequently happens that on the serum-control sheet the amount of complement required for 50 % haemolysis in row 12 (diluent alone) is greater than in the rows immediately above. This is probably due to lack of protective serum, and the unit is calculated on the value in row 11. In the main test any fixation occurring in excess of the calculated non-specific fixation is presumed to be significant.

## RESULTS

The six influenza strains, WS, Barratt, PR8, Shope, Lee and Crawley were compared. To illustrate this comparison the result of the titration of all the antigens against the Lee antiserum is shown in Table 6, and the logarithms of the units fixed have been plotted against the serum dilutions in Fig. 5.

Each curve is now plotted on stout graph paper, the scale in this case being 18 mm. for the abscissal units and 10 mm. for the units of the ordinate, using 0.0 as a base-line. The area under each curve is estimated by cutting out and weighing the pieces of paper (Table 6, i). This weight in mg. is converted into

Table 5. Calculation of the non-specific complement fixation of a standard antigen

| Serum dilutions | Serum control            |                | Calculated non-significant fixation |                |                  |                  |                          |
|-----------------|--------------------------|----------------|-------------------------------------|----------------|------------------|------------------|--------------------------|
|                 | $\log_{10} \text{mm.}^3$ | $\text{mm.}^3$ | $a + 0.2$                           | $\text{mm.}^3$ | $\log_{10}(y-x)$ | $\log_{10}(y-x)$ | $\log_{10} \text{units}$ |
| 1 in            | (a)                      | (x)            | (b)                                 | (y)            | (y-x)            | (z)              | (z-2.9)                  |
| 2               | 1.7                      | 0.5            | 1.9                                 | 0.8            | 0.3              | 1.48             | 0.58                     |
| 4               | 1.4                      | 0.25           | 1.6                                 | 0.4            | 0.15             | 1.18             | 0.28                     |
| 8               | 1.2                      | 0.16           | 1.4                                 | 0.25           | 0.09             | 2.95             | 0.05                     |
| 16              | 1.1                      | 0.13           | 1.3                                 | 0.20           | 0.07             | 2.84             | —                        |
| 32              | 1.0                      | 0.10           | 1.2                                 | 0.16           | 0.06             | 2.78             | —                        |
| 64              | 2.9                      | 0.08           | 1.1                                 | 0.13           | 0.05             | 2.70             | —                        |
| 128             | 2.9                      | 0.08           | 1.1                                 | 0.13           | 0.05             | 2.70             | —                        |
| 256             | 2.9                      | 0.08           | 1.1                                 | 0.13           | 0.05             | 2.70             | —                        |
| 512             | 2.9                      | 0.08           | 1.1                                 | 0.13           | 0.05             | 2.70             | —                        |
| —               | 2.9                      | —              | —                                   | —              | —                | —                | —                        |

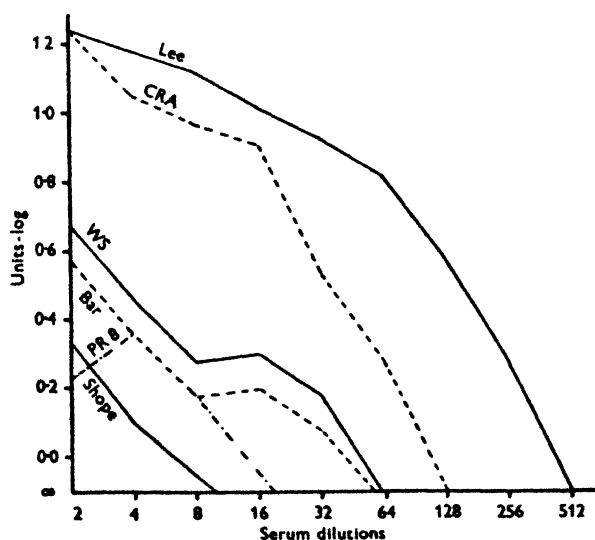


Fig. 5. Lee immune serum titrated with six standard antigens. From data in Table 6.

$\text{cm.}^2$  by dividing by the weight of  $1 \text{ cm.}^2$  of the paper used, here 11.5 mg. (Table 6, ii). All areas less than the area of the calculated non-specific fixation are disregarded, the rest are expressed as fractions of the homologous area (Table 6, iii, which is ii divided by 120). The areas are reproducible to within about 5–10 %, and the relative areas with different antigens are not affected by slight differences in the titre of different pools of antiserum.

The comparison of all the antigens with antisera prepared against them is

shown in Table 7*a*. It will be seen that the cross-reactions are not reciprocal, and therefore to show the relationship between the strains the two fractions are summed and divided by 2 (Table 7*b*). For example, the cross-reactions

Table 6. *Complement fixation of Lee immune serum with six standard antigens. Calculation of index numbers*

| Serum dilution            | Log units  |       |         |       |       |        |         |
|---------------------------|------------|-------|---------|-------|-------|--------|---------|
|                           | Calculated | PR 8  | Barratt | WS    | Shope | Lee    | Crawley |
| 1:2                       | 0.57       | 0.23  | 0.57    | 0.67  | 0.33  | 1.24   | 1.24    |
| 1:4                       | 0.36       | 0.36  | 0.36    | 0.46  | 0.10  | 1.18   | 1.05    |
| 1:8                       | 0.18       | 0.18  | 0.18    | 0.28  | —     | 1.12   | 0.97    |
| 1:16                      | —          | —     | 0.20    | 0.30  | 0.05  | 1.02   | 0.91    |
| 1:32                      | —          | —     | 0.08    | 0.18  | —     | 0.93   | 0.53    |
| 1:64                      | —          | —     | —       | —     | —     | 0.82   | 0.28    |
| 1:128                     | —          | —     | —       | —     | —     | 0.58   | —       |
| 1:256                     | —          | —     | —       | —     | —     | 0.28   | —       |
| 1:512                     | —          | —     | —       | —     | —     | —      | —       |
| 1:1024                    | —          | —     | —       | —     | —     | —      | —       |
| (i) mg.                   | 164        | 132   | 226     | 313   | 50    | 1381   | 914     |
| (ii) cm. <sup>2</sup>     | 14.26      | 11.48 | 19.65   | 27.21 | 4.35  | 120.00 | 79.50   |
| (iii) (ii) divided by 120 | —          | —     | 0.16    | 0.23  | —     | 1.0    | 0.66    |

of PR 8 and Barratt in Table 7*a* are 0.55 and 0 so that the arithmetic mean is 0.28. It is clear that all of the strains examined are different, though Lee and Crawley are closely similar and WS and Barratt are fairly closely related. Apparently there is some slight relationship between the WS strain and the two strains of Influenza B virus.

Table 7(*a*). *Comparison of six strains of influenza virus. Degree of complement fixation expressed by an index number, homologous = 1.0*

| Antigens | Sera |      |      |       |      |      |
|----------|------|------|------|-------|------|------|
|          | WS   | BAR  | PR 8 | Shope | Lee  | CRA  |
| WS       | 1.0  | 0.53 | 0.61 | 0.57  | 0.23 | 0.25 |
| BAR      | 0.63 | 1.0  | 0.55 | 0.57  | 0.16 | 0    |
| PR 8     | 0.26 | 0    | 1.0  | 0.14  | 0    | 0    |
| Shope    | 0    | 0    | 0.20 | 1.0   | 0    | 0    |
| Lee      | 0    | 0    | 0    | 0.14  | 1.0  | 0.84 |
| CRA      | 0    | 0    | 0    | 0.14  | 0.66 | 1.0  |

Table 7(*b*). *Comparison of six strains of influenza virus. Arithmetic mean of cross-reactions*

| Antigens | Sera |      |      |       |      |      |
|----------|------|------|------|-------|------|------|
|          | WS   | BAR  | PR 8 | Shope | Lee  | CRA  |
| WS       | 1.0  | 0.58 | 0.44 | 0.28  | 0.11 | 0.12 |
| BAR      | —    | 1.0  | 0.28 | 0.28  | 0.08 | 0    |
| PR 8     | —    | —    | 1.0  | 0.22  | 0    | 0    |
| Shope    | —    | —    | —    | 1.0   | 0.07 | 0.07 |
| Lee      | —    | —    | —    | —     | 1.0  | 0.75 |
| CRA      | —    | —    | —    | —     | —    | 1.0  |

By similar methods the Barratt 1947 A strain was compared with the Williams strain isolated during the same epidemic, and also with the Rhodes strain isolated in New York in 1947 (Table 8). The three strains are identical.

Table 8. *Comparison of three strains of influenza virus isolated in 1947.*  
*Arithmetic mean of cross-reactions*

|     | BAR | WIL  | RHO  |
|-----|-----|------|------|
| BAR | 1.0 | 0.97 | 0.97 |
| WIL | —   | 1.0  | —    |
| RHO | —   | —    | 1.0  |

## DISCUSSION

In the comparison of a number of strains of influenza virus it has not been possible to demonstrate strictly reciprocal cross-reactions. Because of this it is unlikely that there is a mosaic of antigens variously represented in the individual strains.

The interpretation of cross-reactions is bound to remain hypothetical until chemical details are known, but it seems reasonable to regard the influenza strains as a number of related but in most cases not identical structures, and the cross-reactions as due to the diversity of antibodies produced against the configuration as a whole (cf. Landsteiner, 1945).

The relationships between the strains examined may be understood by considering a number of theoretical strains. A strain  $\alpha$  is presumed to be identical with a strain  $\beta$  when the standard  $\alpha$  antigen gives with the  $\beta$  antiserum the homologous serum titre—and vice versa. Under such circumstances the amounts of complement fixed maximally will necessarily be the same in both cases, here chosen at 1.0 mm.<sup>3</sup> When strains are entirely dissimilar the zone of fixation falls within the calculated non-significant fixation.

Of strains which are similar but not identical there are two groups. In the first group, strain  $\gamma$  is similar to but not identical with strain  $\beta$  when the standard  $\gamma$  antigen is matched with the  $\beta$  anti-serum and the serum titre is less than the homologous titre. This is taken to mean that only some of the antibodies are of a sufficiently good fit to react with the antigen. Owing to the large excess of serum antibodies available there are still sufficient of these antibodies at a serum dilution of 1/2 to enable the antigen to fix the standard 1.0 mm.<sup>3</sup> of complement (Fig. 6, BAR).

In the second group, strain  $\delta$  is even more unlike the  $\beta$  strain, since not only are the available antibodies in the  $\beta$  serum merely a part of those with which the homologous antigen reacts, as shown by the lower serum titre, but also the maximal complement fixed is less than 1.0 mm.<sup>3</sup>, suggesting that the  $\delta$  antigen is heterogeneous and that only certain elements are able to react with certain components of the  $\beta$  antibody population (Fig. 6, PR8).

This distinction as to whether the whole of the standard amount of complement is fixed or not might have been used as a method of classification within the A and B strains. But as the cross-reactions are not reciprocal we have had to accept the mean of the observed cross-reactions.



Friedewald (1944) has studied the antigenic composition of influenza virus by means of antibody absorption tests. However, the value of this difficult technique seems rather doubtful if one considers the cross-reactions as being due to diversity of antibodies produced against the configuration as a whole.

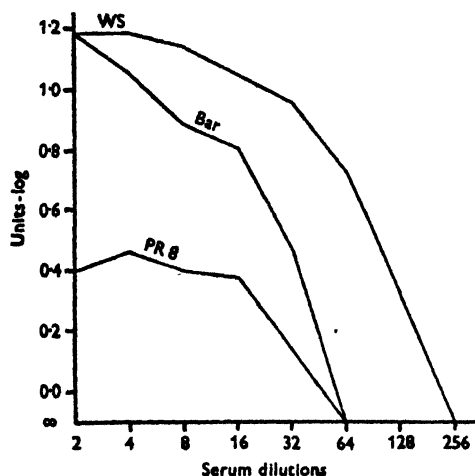


Fig. 6. WS immune serum titrated with three standard antigens to show the fixation of a closely similar strain (BAR) and the fixation of a slightly related strain (PR 8).

For two heterologous strains will only react with the same group of antibodies if they are themselves identical—a fact which can be demonstrated in simpler ways.

We have not attempted a classification of the influenza virus strains—a task which would demand the detailed examination of a large number of strains. The method proposed has, however, enabled us to compare strains with accuracy, and paves the way for a useful classification.

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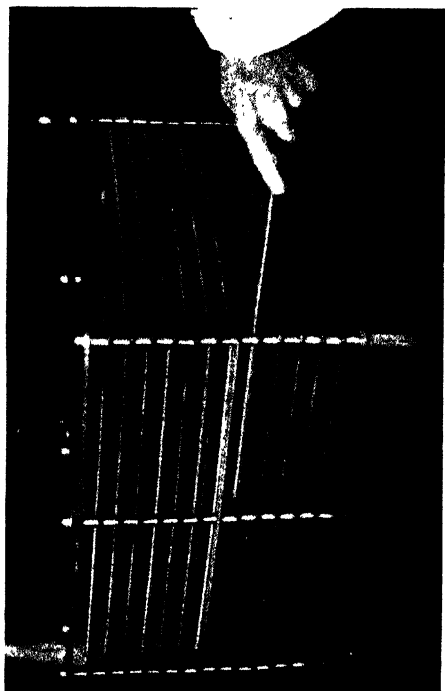


Fig. 2



Fig. 1

Fig. 3

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#### EXPLANATION OF PLATE

- Fig. 1. Technique for complement fixation: setting out drops on a Perspex sheet.
- Fig. 2. Technique for complement fixation: rack to hold 12 Perspex sheets.
- Fig. 3. Technique for complement fixation: to show the method of reading the test. Where the erythrocytes have not been haemolysed they have collected into a central dot

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## The Use of Nile Blue in the Study of Tetrathionase Activity

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**SUMMARY:** Experiments by the Thunberg tube technique have shown that tetrathionate can oxidize reduced Nile blue in the presence of tetrathionate-adapted cells of a Gram-negative coliform organism but not in the presence of cells lacking a developed tetrathionase system, viz. unadapted cells of the same organism and cells of *Shigella sonnei*. Concentrations of tetrathionate of the order of  $m/3200$  and slight tetrathionase activity can be detected by the Thunberg tube technique, which may be useful also in investigating the effect of certain physical and chemical agents on the tetrathionase system. It appears that tetrathionate can act as a hydrogen acceptor for organisms capable of reducing it.

Knox, Gell & Pollock (1948) showed that certain organisms can reduce tetrathionate in the presence of a hydrogen donator, and it has been suggested that tetrathionate acts as a hydrogen acceptor alternative to oxygen for those organisms that are able to reduce it (Knox *et al.* 1948; Pollock & Knox, 1948; Knox, 1945). Quastel & Whetham (1924) showed that under anaerobic conditions, in the presence of a washed suspension of *Bacterium coli*, fumarate inhibited the reduction of methylene blue and reoxidized reduced methylene blue. Quastel, Stephenson & Whetham (1925) showed that nitrate acted in a similar way, and Green, Stickland & Tarr (1934) performed similar experiments with other redox indicators. It is accepted that the reoxidation, of a reduced indicator under anaerobic conditions, is evidence of the presence of some substance or system capable of acting as a hydrogen acceptor alternative to oxygen. The extent to which a reduced redox indicator is reoxidized, however, depends on the relative rates at which it is being oxidized and reduced by the systems present in the preparation. The 'tetrathionase' system, which is an adaptive enzyme system (Knox & Pollock, 1944), transfers hydrogen from a suitable hydrogen donator to tetrathionate and almost certainly consists of a complex series of enzymes. In this investigation only that part of the system which transfers hydrogen from reduced Nile blue to tetrathionate has been studied. The experiments described here show that tetrathionate will reoxidize reduced Nile blue in the presence of a suspension of cells adapted to reduce tetrathionate, but not in the presence of cells which do not reduce tetrathionate.

### EXPERIMENTAL

#### *Methods*

**Organisms.** The organisms used were: (1) a non-pathogenic Gram-negative coliform organism of the intermediate type I group, labelled '1488'; this organism was obtained from Dr M. R. Pollock and had been used by Knox *et al.* (1948) in their work on the selective action of tetrathionate, and by

Pollock (1946) in his work on nitratase; (2) a strain of *Shigella sonnei*, as an example of a non-reducer of tetrathionate.

**Preparation of suspensions.** Normal (unadapted) cell suspensions of '1433' and the suspension of Sonne cells were prepared by inoculating 3 ml. of 6 hr. tryptic heart-broth cultures on to 200 ml. of tryptic heart agar in Roux bottles. The Roux bottles were incubated at 37° for 16 hr., and the cells were washed off in quarter-strength Ringer solution. The cells from each Roux bottle were centrifuged, washed once, and made into a thick suspension in 10 ml. quarter-strength Ringer solution.

Adapted cell suspensions of '1433' were prepared by growing the organisms in Roux bottles containing M/50 tetrathionate, M/50 mannitol and M/5 phosphate buffer (pH 7.6) in tryptic heart agar and treating in the same way (Knox & Pollock, 1944). The dry weight of all these suspensions was of the order of 15–20 mg./ml.

**Preparation of Thunberg tubes.** The experiments were performed in evacuated Thunberg tubes placed in a thermostatically controlled water-bath at 37°. The following mixture was placed in the main part of each tube: 0.5 ml. Nile blue M/1600; 0.5 ml. sodium lactate M/2; 0.5 ml. phosphate buffer (pH 6.4) M/7.5; 0.5 ml. bacterial suspension. The mixture was buffered at pH 6.4 because Nile blue was more readily decolorized at this pH than at pH 7.6, and the activity of tetrathionase falls off rapidly at pH levels lower than 6.4 (Pollock & Knox, 1943). Volumes (0.5 ml.) of various concentrations of tetrathionate were placed in the hollow stoppers of the Thunberg tubes and the tubes closed and evacuated by water pump. After the Nile blue had been decolorized by the action of the cell suspension the tetrathionate was tipped into the mixture. Sodium tetrathionate prepared by the method described by Sander (1915) was used; it contained two molecules of water of crystallization and no detectable iodide, thiosulphate, sulphate or sulphite (Knox, 1945). In a few experiments tetrathionate was added to the mixture in the Thunberg tube before the Nile blue was reduced, to see if it would inhibit the reduction of the dye. A few similar experiments were performed with methylene blue as an indicator.

### Results

**Reoxidation of reduced Nile blue.** Table 1 shows the results of an experiment using adapted and unadapted cells of '1433' and a Sonne suspension. It can be seen that tetrathionate caused an immediate reoxidation of the Nile blue in the presence of the adapted cells of '1433', while with the other two suspensions the addition of tetrathionate produced only a pale blue colour. The time taken for this colour to disappear was, however, slightly longer with the unadapted cells of '1433' than with the Sonne suspension.

Experiments to find the smallest concentration of tetrathionate that would reoxidize reduced Nile blue completely in the presence of adapted cells of '1433' are recorded in Table 2. It can be seen that tetrathionate down to a final concentration of M/3200 completely reoxidized the Nile blue used, and that an even lower concentration of tetrathionate delayed the disappearance

of the pale blue colour which appeared on adding the contents of the stopper.

The effect of diluting a suspension of adapted cells of '1488' is shown in Table 3. Twofold dilutions of adapted cells were added to 0.3 ml. of a Sonne suspension in a Thunberg tube containing the usual mixture, the Sonne suspension being used to ensure rapid reduction of the Nile blue. The tetrathionate was added when the Nile blue was reduced, and the resulting colour

Table 1. *Effect of adding tetrathionate to reaction mixtures containing various washed cell suspensions*

The main part of each Thunberg tube contained: 0.5 ml.  $M/2$  sodium lactate; 0.5 ml.  $M/7.5$  phosphate buffer pH 6.4; 0.5 ml.  $M/1600$  Nile blue; 0.5 ml. of cell suspension.

| Cell suspension  | Final concentration of tetrathionate after mixing | Time taken to decolorize Nile blue (min.) | Deepest colour reached after adding contents of stopper | Time taken for colour to disappear again completely (min.) |
|------------------|---|---|---|--|
| '1433' adapted   | Nil   | $6\frac{1}{2}$                            | Pale blue   | 1-1 $\frac{1}{2}$  |
| '1433' adapted   | $M/100$   | $6\frac{1}{2}$                            | Deep blue   | > 240  |
| '1433' unadapted | $M/100$   | $7\frac{1}{2}$                            | Pale blue   | $3\frac{1}{2}$   |
| Sonne            | $M/100$   | 6   | Pale blue   | 1 $\frac{1}{2}$  |

Table 2. *Effect of adding various concentrations of tetrathionate to reaction mixtures containing tetrathionate-adapted cells*

The main part of each Thunberg tube contained: 0.5 ml.  $M/2$  sodium lactate; 0.5 ml.  $M/7.5$  phosphate buffer pH 6.4; 0.5 ml.  $M/1600$  Nile blue; 0.5 ml. of adapted '1488' cells.

| Final concentration of tetrathionate after mixing | Time taken to decolorize Nile blue (min.) | Deepest colour reached after adding contents of stopper | Time taken for colour to disappear again completely |
|---|---|---|---|
| Nil   | $6\frac{1}{2}$                            | Pale blue   | 1-1 $\frac{1}{2}$ min.                              |
| $M/100$   | $6\frac{1}{2}$                            | Deep blue   | Still deep blue at 4 hr.                            |
| $M/200$   | 6   | Deep blue   | 1 hr.   |
| $M/400$   | 6   | Deep blue   | 30 min.   |
| $M/800$   | 6   | Deep blue   | 11 min.   |
| $M/3200$  | 6   | Deep blue   | $6\frac{1}{2}$ min.                                 |
| $M/6400$  | $6\frac{1}{2}$                            | Pale blue   | 5 min.  |

and the time for it to disappear were noted. It can be seen from Table 3 that as the adapted cell suspension of '1488' was diluted the Nile blue was not fully reoxidized, and the time taken for the colour to disappear again completely was lengthened. On further dilution the time taken for the dye to be reduced completely again became shorter until in the last tube it was very little longer than in tube 2 which contained distilled water in place of adapted '1488' cells.

Experiments were performed with unadapted cells of '1488' and with a Sonne suspension, using various concentrations of tetrathionate in the stopper. Complete reoxidation of the Nile blue did not occur with either of these suspensions; only the usual pale blue colour appeared when the contents of the stopper were added to the mixture after initial reduction of the dye. With

the Sonne suspension the time taken (*c.* 1.5 min.) for this pale colour to disappear again was the same whether water or tetrathionate was added from the stopper. With the unadapted suspension of '1433', however, the time for the colour to disappear was slightly longer (*c.* 3.5 min.) when tetrathionate was added than when water was added (1.5 min.). These differences are very small

Table 3. *Effect of decreasing quantity of adapted cells in the reaction mixture*

Each tube contained: 0.5 ml. *M*/2 sodium lactate; 0.5 ml. *M*/7.5 phosphate buffer pH 6.4; 0.5 ml. *M*/1600 Nile blue; 0.5 ml. suspension; in the stopper 0.5 ml. *M*/80 tetrathionate. Tube 1 contained 0.5 ml. Sonne suspension, the others 0.3 ml. Sonne suspension and 0.2 ml. suspension of adapted cells of '1433' in the dilutions indicated.

| Tube no. | Cell suspension |   | Time taken to decolorize Nile blue (min.) | Deepest colour reached after adding contents of stopper | Time taken to recolorize Nile blue          | Time taken for colour to disappear again completely |
|----------|-----------------|---|---|---|---|---|
|          | Sonne (ml.)     | Dilution of adapted '1433' of which 0.2 ml. was added |   |   |   |   |
| 1        | 0.5             | —   | 7   | Pale blue   | —   | 2½ min.   |
| 2        | 0.3             | Distilled water only                                  | 9   | Pale blue   | —   | 3½–4 min.   |
| 3        | 0.3             | 1/1 (undiluted)                                       | 6   | Deep blue   | 1 min. approx.                              | 65 min.   |
| 4        | 0.3             | 1/2   | 7   | Blue ?deep  | ?not quite fully; > 5 min. to reach maximum | <i>c.</i> 2 hr.                                     |
| 5        | 0.3             | 1/4   | 7½  | Blue  | Not fully; long time to reach maximum       | 2–3 hr.   |
| 6        | 0.3             | 1/8   | 9   | Blue  | —   | Very pale after 25 min.; fully decolorized 40 min.  |
| 7        | 0.3             | 1/16  | 8   | Pale blue   | —   | 18 min.   |
| 8        | 0.3             | 1/32  | 8   | Pale blue   | —   | 8 min.  |
| 9        | 0.3             | 1/64  | 8½  | Pale blue   | —   | 5 min.  |

but may indicate partial reoxidation of the reduced Nile blue by the slight tetrathionase activity of the unadapted cells. Table 3 shows that such tetrathionase activity must be very small when compared to that of adapted cells.

*Enzymic nature of process.* The system responsible for the reoxidation of Nile blue by tetrathionate in the presence of adapted cells of '1433' can be destroyed by heat. The contents of the main part of a Thunberg tube were heated to 65° for 40 min. and allowed to cool after the Nile blue had been reduced and before tetrathionate was added from the stopper. The deep blue colour which appeared when tetrathionate was added to the unheated mixture did not appear when the same concentration of tetrathionate was added to the heated and cooled mixture.

*Delay in reduction of redox indicators.* Experiments were performed in which various concentrations of tetrathionate were added to the mixture of suspension, lactate, buffer and Nile blue in the main part of a Thunberg tube before the tube was evacuated and placed in the water-bath. It was found that even *M*/1600 (final concentration) tetrathionate gave a perceptible delay in the



reduction of Nile blue by adapted cells of '1433' but not by unadapted cells or by a Sonne suspension. Tetrathionate in quite small concentrations also inhibited the reduction of methylene blue by adapted but not unadapted cells of '1433'. Attempts to reoxidize reduced methylene blue with tetrathionate in the presence of adapted cells were not successful.

### DISCUSSION

The experiments performed show that under suitable anaerobic conditions tetrathionate can oxidize reduced Nile blue in the presence of cells adapted to reduce tetrathionate, but not in the presence of cells which cannot reduce tetrathionate. The technique used was capable of detecting tetrathionate in concentrations as low as  $M/3200$ , and of detecting slight tetrathionase activity. The tetrathionase activity of the unadapted cells used here must be very small.

Attempts to inhibit the dehydrogenase systems of the adapted cells while leaving the tetrathionase system intact have so far been unsuccessful. Substances that inhibit the dehydrogenase systems appear to affect the tetrathionase system also, though perhaps rather more slowly. By means of the Nile blue reduction-oxidation technique, however, it may be possible to study the effect of various chemical and physical agents on the tetrathionase system.

I am indebted to Dr R. Knox and Dr M. R. Pollock for much valuable advice and to Mr A. H. Tomlinson who kindly prepared and investigated the pure sodium tetrathionate used.

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## The Control of the Swarming of *Proteus vulgaris* by Boric Acid

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**SUMMARY:** The swarming of *Proteus vulgaris* is inhibited on a heated blood-agar medium containing 0.1 % (w/v) boric acid. This boric acid concentration does not inhibit the growth of many organisms having more exacting metabolic requirements. The medium is equally successful in controlling certain swarming strains of *Pseudomonas pyocyaneus*. This inhibition of swarming is possibly due to the formation of a boron-polysaccharide complex, resinous in character, the formation of which is associated with flagellar disintegration.

The swarming of *Proteus vulgaris* in mixed culture is a continual source of trouble to bacteriologists. A method which could control it without affecting the growth of more delicate organisms would be of great value, e.g. in the isolation of gonococci from the urogenital tract. Kramer & Koch (1931) in a summary comparing existing methods indicated chloral hydrate as one of the most successful inhibitors of swarming. Gillespie (1948) used this substance for the isolation of  $\beta$ -haemolytic streptococci on a blood-agar medium in the presence of *P. vulgaris*.

Lominski & Lendrum (1942) proposed the use of surface-active agents to prevent swarming. Those compounds which they described as most effective, together with others not mentioned by them, proved on test in our hands to be incapable of preventing the swarming of *P. vulgaris* when these substances were incorporated in a heated blood-agar medium. Simple inorganic salts were next examined, especially those having some protein-coagulating action, which might be expected to influence either the formation or the action of flagella. Potash alum was inhibitory in a simple nutrient agar medium and in a heated blood-agar medium containing 10 % blood, but was lethal to more delicate organisms at concentrations sufficient to prevent the swarming of *P. vulgaris*. The active part of potash alum proved to be the aluminium. Accordingly, other members of group 3 of the Periodic Table were investigated. Boron compounds and in particular boric acid were found to be peculiarly effective. Boric acid was therefore incorporated in a heated blood-agar medium containing 10 % blood in concentrations ranging from 1 to 0.0001 % (here and throughout % means % (w/v)). The most satisfactory concentration of boric acid for the inhibition of swarming was 0.1 %, and this concentration did not affect the growth of more delicate organisms. At concentrations of 1 % boric acid there was only moderate growth of *Shigella sonnei*, *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Neisseria gonorrhoeae*. Swarming of some of the twelve strains of *Proteus vulgaris* examined began at dilutions lower than 0.05 % boric acid; other strains did not swarm until a dilution of 0.01 % was reached.

In the standard medium used in this department for the isolation of gonococci from the urogenital tract, a heated blood agar containing 20 % blood, it was found advisable to increase the concentration of boric acid to 0.125 %. The suitability of the boric acid medium for isolating gonococci was estimated by comparing the minimum number of gonococci growing on a heated blood-agar medium with and without boric acid. Using six freshly isolated strains of gonococci suspended in 10 % serum broth, and various dilutions of these suspensions, it was found that both media detected the organism at a dilution of 1000 organisms/ml. when the inoculation was made with a standard 3 mm. loop which contained at that dilution *c.* 70 organisms. The medium was tested extensively in the Venereal Diseases Clinic of the General Infirmary at Leeds.

*The boric acid medium.* The medium was made as follows. A screw-capped bottle of about 550 ml. capacity containing 400 ml. of nutrient agar was placed in the steamer until the agar was melted. The bottle was then transferred to a water-bath at 60° and kept at that temperature for 10 min.; 80 ml. of oxalated horse blood were then added and well mixed. The temperature of the bath was then raised to 75°. When the contents of the bottle had assumed a uniform chocolate colour 12.5 ml. of a 4 % solution of boric acid were added (a 4 % solution of boric acid is self-sterilizing) and well mixed in the medium by rotatory movements of the bottle; plates were then poured. After setting, the plates were kept in the cold-room for use as required; it was found that the medium kept for 4–6 weeks without deterioration.

#### *Technique and results of test*

The method adopted for testing the medium was to inoculate first the control plate and then the boric acid plate with the same swab. This procedure was adopted because it tended to load the results against the boric acid plates. In a parallel examination of 850 specimens fifty-six of the control plates were overgrown by *Proteus vulgaris*. Of the boric acid plates none was overgrown by *P. vulgaris*, although in each case that organism was demonstrable in discrete colonies. The successful isolation of gonococci was obtained in thirty-eight instances on the boric acid medium but only in twenty-six on the control medium. The medium therefore shows promise as a diagnostic aid for the detection of the gonococci in the urogenital tract, and may be similarly useful on other occasions where the swarming of *P. vulgaris* impedes the isolation of desired organisms, e.g. in material from burns.

#### *Possible mechanism of action of boric acid*

The work of Deuel, Neukorn & Weber (1948) suggested a possible explanation for the inhibiting action of the medium on the swarming of *P. vulgaris*. These authors have shown that polyhydroxylic substances, particularly those having a high degree of polymerization, form resinous compounds with aqueous solutions of boric acid even at concentrations low in comparison with those used in the medium described. Electron micrographs of *P. vulgaris* grown on a heated blood-agar medium containing boric acid (Pl. 1, fig. 1) show few

flagella, most of which are broken and detached; cells grown on a similar medium without boric acid show large numbers of undistorted and unbroken flagella. The examination of flagellated bacteria in the electron microscope is complicated by the fact that during mounting and drying the flagella may become detached or broken unless a special technique be adopted. In this case, to avoid damage to the flagella, dilute suspensions were made in filtered distilled water, care being taken to avoid undue shaking and stirring. Small drops of the suspensions were placed on filmed specimen grids. After standing for 5 min. these were drained with a small piece of filter-paper. This method ensured the even distribution of the organisms on the grid, and the rapid drying so obtained minimized distortion and damage to the flagella. The dried grids after washing by flotation on filtered distilled water were shadow-cast with chromium, using the vacuum technique of Williams & Wyckoff (1944). Material in salt solution when placed on the grid is unavoidably accompanied by large amounts of other matter including salt. In order that the structural detail of the material may be clearly seen this extraneous matter must be removed in a way that will not damage the material under examination. Various methods of accomplishing this without affecting the nature and degree of aggregation have been considered by Reed (1946). One of the simplest and most effective is to form a thin layer of a dilute suspension of the material on the filmed specimen grid by using a micro-pipette, and to drain immediately. In such very thin layers the chances of particle aggregation by lateral movement are greatly diminished and the specimens dry rapidly. With most biological material, drying at this stage fixes it in an insoluble state, so that excess of salt can be safely removed by washing before shadow-casting. Figs. 2 and 3 in Pl. 1 were made from material treated in this manner and are clearly free from salt crystals. It was felt that pictures of preparations containing only flagella would prove more instructive for the understanding of the prevention of swarming by boric acid.

#### *Preparation of flagella suspensions*

For the preparation of suspensions of flagella the following technique was adopted. A 24 hr. culture of *Proteus vulgaris* which had been inoculated at the junction of the condensation water and the agar slope was used. This ensured that the culture used was actively swarming. The culture was removed by careful washing with 10 ml. of isotonic saline and the resultant suspension divided into two equal parts. To one part boric acid was added to give a final concentration of 0.1 %. Both suspensions were then incubated for 18 hr. at 37° and then 0.1 ml. of 80 % formaldehyde added. The suspensions were then centrifuged at 7000 G for 20 min., and the resultant clear supernatant fluids examined in the electron microscope. The electron micrographs (Pl. 1, figs. 2, 3) show that the boric acid-treated fluid contains the remains of flagella on whose surfaces are numerous small spherical particles and agglomerations of these particles, of which the average diameter is 250 Å. (fig. 3). In the control fluid the flagella appear to be normal and are not clumped (fig. 2).

*Possible nature of boric acid-polyhydroxylic complex*

It may well be that the particles observable in Pl. 1, fig. 8, represent the protein fraction of the flagella, and that the thinner layer which surrounds them and appears to link them is a complex of the type mentioned by Deuel *et al.* (1948). If this is so then the clumping of the flagella is more easily explicable, as these complexes are known to be of a viscous nature. Deuel (1948) states that the presence of even minute traces of these substances may be detected by viscosimetric measurements. It was found that the fluid treated with boric acid showed a much greater time of flow than the fluid of the control tube, and than negative controls made from uninoculated agar slopes. The same viscosimeter (Ostwald type) was used in each case and was thoroughly cleaned after each reading. The times compared were the mean of ten readings for each specimen; all readings were made at 18°. The increase in time of flow of the specimen treated with boric acid was of the order of 9%.

Deuel (1948) states that the action of acid or fructose destroys these complexes. Fluid obtained, as already mentioned, from a tube which had been treated with boric acid was acidified. To 10 ml. of clear supernatant fluid 1 ml. of 0.1 N-HCl was added, and after 1 hr. specimens were placed on filmed grids. Examination in the electron microscope showed numerous small particles (Pl. 1, fig. 4), slightly smaller than those observable in Pl. 1, fig. 3, which it has been suggested were covered by a thin layer of some less dense material and which is not observable in Pl. 1, fig. 4. These particles are not clumped. This lack of clumping may be due to the destruction by the acid of a complex of the nature already mentioned. Viscosimetric examination showed that the time of flow of the acid-treated specimen approached that of the blank controls.

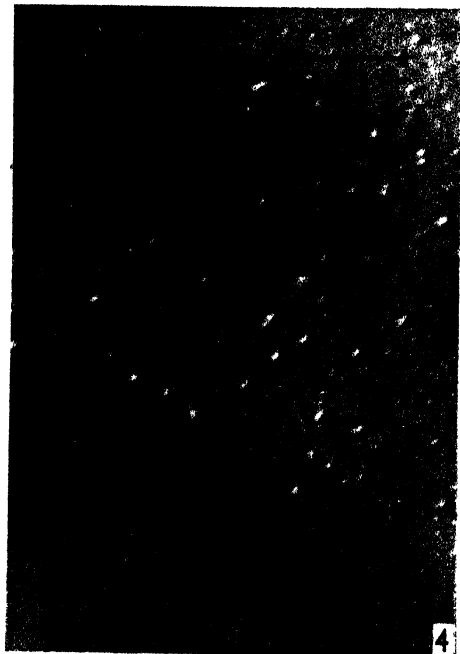
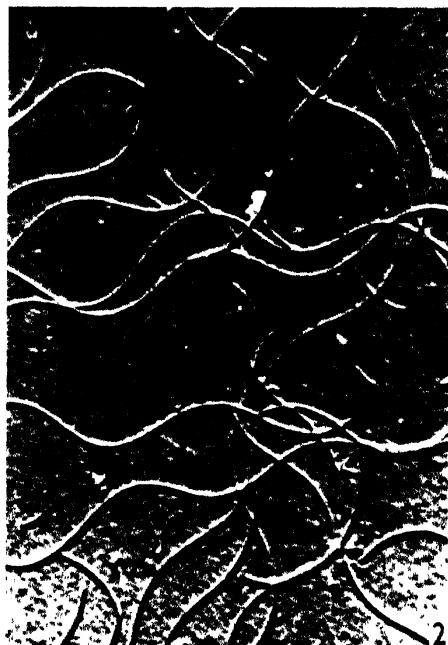
*Effect of copper sulphate and lowering of pH*

Other inorganic salts that have been used for inhibiting the swarming of *P. vulgaris* were similarly examined. Copper sulphate when substituted for boric acid at a final concentration of 0.05% had no detectable effect on free flagella. To obviate the possibility that the boric acid phenomenon might be due to a lowering of pH value a specimen was prepared using the same technique but adding 1 ml. of 0.1 N-HCl. In the electron microscope no change from the normal was observed.

## DISCUSSION

Although it would be very difficult to prove conclusively that the action of boric acid on the flagella of *Proteus vulgaris* and *Pseudomonas pyocyanea* is due to the formation of a boron complex of the type suggested, the evidence presented points in that direction. It is probable that the differences between the flagella in fig. 1 and fig. 8 of Pl. 1 are due to the very much greater surface area exposed to the action of boric acid in the flagella of fig. 8. Although other interpretations of the electron micrographs are possible the viscosimetric com-





Figs. 1-4

J. A. SYKES & R. REED. THE CONTROL OF THE SWARMING OF *PROTEUS VULGARIS* BY BORIC ACID.  
PLATE 1

parisons favour the theory that a boron complex of the nature described is formed. Assuming this, then the flagella probably consist of aggregates of protein molecules bound and surfaced by a polyhydroxylic substance.

We wish to thank Prof. J. W. McLeod for suggesting the problem, allowing one of us (J.A.S.) to use the facilities of his department, and for his helpful advice; Dr R. Lees of the Venereal Diseases Clinic of the General Infirmary, Leeds, for his co-operation in the trial of the boric acid medium; Prof. W. T. Astbury and Mr A. Millard of the Department of Biomolecular Structure for their co-operation and advice in the production of the electron micrographs.

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#### EXPLANATION OF PLATE

- Fig. 1. Electron micrograph of *Proteus vulgaris* taken from a heated blood-agar medium (10 % blood) containing 0.1 % boric acid after 24 hr. incubation at 37°. Magnification  $\times 33,000$ .  
Fig. 2. Electron micrograph of supernatant fluid from control suspension showing undistorted flagella of *Proteus vulgaris*. Magnification  $\times 33,000$ .  
Fig. 3. Electron micrograph of supernatant fluid from boric acid-treated specimen, showing alteration in appearance of flagella. Magnification  $\times 33,000$ .  
Fig. 4. Electron micrograph of supernatant fluid from specimen treated with boric acid after further treatment with HCl. Magnification  $\times 24,000$ .

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## Auxanographic Techniques in Biochemical Genetics

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**SUMMARY:** Details are given of the 'auxanographic' method for identifying growth-factor requirements of micro-organisms, and particularly of mutant strains differing from a parent strain in having additional growth-factor requirements. Cells are distributed in a nutritionally deficient agar medium which is allowed to set in a Petri dish. Possible required nutrients are then spotted on the agar surface, thus enabling the corresponding exacting mutants to grow at those localities. The technique is only a labour-saving device which can be extremely efficient in shortening the preliminary steps in the work. It can also be applied to other uses, such as the identification of competitive interactions between nutritives.

Sixty years ago, in 1889, Beyerinck (Beijerinck) described a technique, simple and efficient, for the study of the nutritional requirements of micro-organisms. The 'auxanographic' technique, as he called it, has gone almost unnoticed; the present author became aware of Beyerinck's paper only after having redeveloped the technique, developed it for biochemical genetics, and used it for several years (Pontecorvo, 1947). Its efficiency in saving labour and its many uses make worthwhile a description of its applications to the study of the genetics of micro-organisms and certain other problems.

On a densely seeded agar plate of a medium lacking a required growth factor for a given micro-organism, no visible growth can take place. If, however, we place at a point of the agar surface a minute amount of the missing growth factor, this will diffuse out, and on incubation growth will take place in a circular area centred on that point. Instead of a growth response other kinds of visible response can be used; Beyerinck suggested, for instance, luminescence, secretion of a pigment, etc.

The technique can be used in reverse, as, indeed, it has been used in Heatley's cylinder assay for penicillin and its numerous variations. If we place a minute amount of an inhibitory substance at a point on the agar surface of a seeded nutrient medium, a circular clear area without growth, surrounded by a general background with growth, will remain after incubation.

The first kind of test, based on the promotion of growth, could perhaps be termed 'positive' auxanography; the second, based on inhibition of growth, 'negative' auxanography. The two are sometimes used in combination, for instance, for investigating the mode of action of a metabolite analogue (see below), and are suitable for quantitative as well as qualitative tests. An example of quantitative positive auxanography is the plate assay for growth factors (see below), and an example of quantitative negative auxanography is the cylinder assay for penicillin. Qualitative negative auxanography has been used by a number of authors especially for detecting the presence of antibiotics in culture filtrates and their spectrum of activity. Qualitative positive auxanography has been extensively used by the present author for a quick

characterization of the nutritional requirements of mutant strains as compared with those of a parent strain; the present paper describes mainly the technique of qualitative positive auxanography as used for this purpose.

*Qualitative positive auxanography for the screening of nutritional mutants*

With Beadle's technique, devised originally for *Neurospora*, but subsequently adapted to other moulds, bacteria and yeasts, nutritionally exacting mutant strains are identified as such in so far as they cannot grow on the simplest chemically defined medium ('minimal') sufficient for growth of the parent strain, but can grow on a complex medium ('complete') containing a large variety of known and unknown organic compounds. After identification, isolation, and genetical or other tests, a mutant has to be characterized, i.e. a search is made for the simplest growth factor, or combination of growth factors, which will make growth possible when added to the minimal medium. In other words, there must be identified the growth factor, or combination of growth factors, present in the complete medium but not in the minimal medium, which account for the growth of the mutant on the former and not on the latter. It is in this characterization of the growth requirements of a mutant, particularly in the earlier stages, that the auxanographic tests have proved to be invaluable.

## EXPERIMENTAL

The technique used in the case of *Aspergillus nidulans* will be given here; only minor variations are required for use with other micro-organisms. *A. nidulans* is a filamentous fungus, most naturally occurring strains of which grow on a minimal medium made up as follows: 25 g. glucose; 15 g.  $\text{NaNO}_3$ ; 1.3 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.3 g. KCl; 3.8 g.  $\text{KH}_2\text{PO}_4$ ; 10.0 mg.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.0 mg.  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; distilled water 2500 ml. This medium is solidified with 1.5% agar. The agar is purified by washing in running water for at least 2 hr.

The normal strain will, of course, grow equally well or better on complex media supplying many additional substances. One of these 'complete' media is the following: glucose, 10 g.; peptone, 10 g.; 'Yeastrel', 5 g.; tap water 1000 ml. Any brand of peptone or of yeast extract can be used.

With each mutant strain, obtained by one of the various techniques available, a search is made for the substance (or substances) present in the complete medium, which is necessary as a supplement to the minimal medium to make possible the growth of the mutant on it.

Petri dishes of minimal medium agar are inoculated by adding to the cooled (50°) molten agar a heavy inoculum of the strain to be tested. This inoculum should secure potential uniform growth in the whole plate; i.e. at least, 1,000,000 conidia/Petri dish. The inoculated plate is incubated for 6 hr. or more to allow the spores to germinate, and after that, minute amounts of various substances are placed in marked positions on the agar surface; a straight platinum wire with which each substance has been just touched provides enough material. When one of these substances, or mixtures of substances,

is capable of promoting the growth of the mutant, after further incubation for 24 hr. a circular area of growth appears around the position where the successful substance has been put. For most purposes the substances to be tested need not be sterile; the high concentration at the point of inoculum usually takes care of that.

The auxanographic test is carried out in successive steps by using arrays of substances which progressively restrict the field of search. In routine work for the characterization of mutants it has been found useful to proceed as follows. In the first step three materials: vitamin-free casein hydrolysate (Ashe Laboratories Ltd.), supplemented with tryptophan; yeast nucleic acid (British Drug Houses Ltd.); and 'Yeastrel', are tested simultaneously on the same Petri dish. The yeast extract is used in even smaller amounts than the other two ingredients (see above). Two or three Petri dishes are usually enough to give an indication of consistent, as distinct from accidental, response.

According to the results obtained from the first step, the second step will be in one or other direction. For instance, response to casein acid-hydrolysate, but not to yeast nucleic acid, and a very small response to yeast extract, will suggest the use of various amino-acids in the second step. On the other hand, good response to yeast extract, but not to yeast nucleic acid nor to casein hydrolysate points to a need for vitamins of the B group.

It is cheaper and almost as efficient to use in the first step the materials mentioned rather than to follow the more precise and reliable method of using only mixtures of known chemical compounds, and omitting one or more components in successive tests. After the first step, the successive ones are, of course, carried out with known mixtures of chemically defined substances or with single substances. Once a particular substance has been identified as capable of promoting growth of a mutant, the further tests for establishing whether this is the only one capable of doing so have to be carried out with the usual biochemical procedures, though the auxanographic test can go a long way towards the identification of the simplest effective substance.

In view of the particular interest which, at the present moment, centres on the biosynthesis of polypeptides, the use in the first auxanographic test of enzymic partial digests of vitamin-free casein at the same time as casein acid-hydrolysate, may be rewarding. Response to the former, but not to the latter, would indicate a requirement for a polypeptide.

An example of the characterization of a mutant of *Aspergillus nidulans* is given in Pl. 1, figs. 1-2. The mutant turned out to be one requiring for growth the addition of lysine to the minimal medium. Fig. 1 shows the response to the three materials mentioned before. It is clear that casein acid-hydrolysate is effective, while yeast nucleic acid and yeast extract are not. Fig. 2 shows the subsequent test, i.e. that of a number of amino-acids suggested by the results of the first test; there is a clear response to one of them (lysine) but not to any of the others. A different mutant gave good response to both casein acid-hydrolysate and yeast extract. A response of this kind suggests the effectiveness of both a vitamin of the B group and an amino-acid. Among other compounds, nicotinic acid and tryptophan were tested in the second-stage auxanographic

test and were both found to be effective. In a third test some of the presumed intermediates and precursors of tryptophan and nicotinic acid were tested, and the mutant was found to respond to anthranilic acid, indole, kynurenine and nicotinic acid (Pl. 1, fig. 8).

The auxanographic test can reveal also cases of double requirements (i.e. two substances simultaneously required for growth) and cases of inhibition of the biological activity of one substance by another. An example of the first kind is given in Pl. 1, figs. 4, 5, which show the simultaneous effect of aneurin and thiosulphate on a mutant of *A. nidulans* which requires both because of two independent mutational changes. The test can be done either, as fig. 4 shows, by using cylinders containing the two solutions, or by placing the crystals of the two substances directly on the agar surface. In the latter case, a further refinement is that of streaking the two substances at right angles; the resulting area of growth will be determined by the co-ordinates of the concentrations of both substances which are compatible with growth (Pl. 1, fig. 5).

Another way in which the auxanographic test can be used is in the identification of the inhibitory action of one substance on the biological activity of another one. Fig. 6 (Pl. 1) shows a test in which the mentioned lysine-requiring mutant of *A. nidulans* was tested for possible inhibition by arginine. Arginine and lysine were streaked on the plate at right angles as uniformly as possible along the length of the streaks. The resulting shape of the area in which growth took place indicated the mode of inhibition of lysine by arginine. It is clear that over a large range of concentrations, the inhibitory level of arginine was independent of the concentration of arginine, but dependent on the ratio of the concentration of arginine to that of lysine. In other words, the approximately straight line which limited the area of growth from that of inhibition indicated that the type of interaction between the two substances was probably a competitive one. It is clear that in many cases competitive interaction may be discovered by this simple procedure, which requires only one or a few Petri dishes and overnight incubation.

#### *Plate assays for growth factors*

The use of positive quantitative auxanography for the assay of growth factors has been only recently developed in industrial laboratories on a practical scale, though it had been implicit in the auxanographic tests described by the author in the last few years (Pontecorvo, 1947). Fig. 7 (Pl. 1) shows a plate assay carried out with a mutant of *A. nidulans* which requires biotin. In this case the method used was that of soaking standard small disks of sterile filter-paper in solutions containing various concentrations of biotin and placing them on to the surface of the agar. Cylinders, instead of disks of filter-paper, can obviously be used, as well as any other of the various devices developed for plate assays of antibiotics. The great advantage of positive auxanography as a method of microbiological assay lies in the possibility of using moulds, and especially mutants of moulds, which are at present being produced in large numbers in laboratories all over the world, without having to weigh the mycelium to estimate the amount of growth.

## CONCLUSION

The auxanographic method, adapted to various needs and various organisms, can be of invaluable help in many directions, but especially in the characterization of the nutritional requirements of micro-organisms. It is, obviously, only a relatively crude method, and its aim is only that of decreasing the amount of labour involved in restricting the field of search for exact nutritional requirements. It is probably adaptable to the identification of co-factors required by lysins to lyse cells, by bacteriophages to become adsorbed on to the host, and by bacterial or other enzymes to give colour, or other visible, reactions.

I am indebted to Mr E. Forbes for invaluable technical assistance. Part of the work was supported by a grant for special research from the Department of Scientific and Industrial Research.

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 PONTECORVO, G. (1947). The genetical control of nutritional requirements in micro-organisms and its application to microbiological assays. *Proc. Nutrit. Soc.* **5**, 182.

## EXPLANATION OF PLATE

- Fig. 1. First step in the auxanographic test; effect of casein acid-hydrolysate (plus tryptophan), yeast nucleic acid and yeast extract. The figure shows a mutant of *Aspergillus nidulans* responding to casein hydrolysate.  
 Fig. 2. Second step. The same mutant, tested for response to various amino-acids; response only to lysine.  
 Fig. 3. A mutant of *A. nidulans* which responded to both casein acid-hydrolysate and yeast extract, is tested for response to possible intermediates in the synthesis of nicotinic acid.  
 Fig. 4. A 'double' mutant of *A. nidulans* requiring both aneurin and thiosulphate. The two substances diffuse out from the cups and growth takes place where the concentration of both is above a certain level.  
 Fig. 5. Same test as fig. 4, but each substance is laid along one of the two dark lines.  
 Fig. 6. The lysine-requiring mutant, of figs. 1 and 2, is tested for a possible competitive inhibition by arginine. The lysine was laid along a streak at right angles to the arginine streak.  
 Fig. 7. Plate assay of biotin with a biotin-requiring mutant of *A. nidulans*; concentrations of biotin in  $\mu\text{g./ml.}$

(Received 31 May 1948)

ADDENDUM (18 November 1948). I am indebted to Professor Gäumann for calling my attention to two papers reporting extensive use of the auxanographic technique.

- GÄUMANN, E. (1921). Over een bakteriële vaatbundelziekte der bananen in Nederlandsch-Indië. *Mededeelingen van het Instituut voor Plantenziekten*, No. 48. Batavia.  
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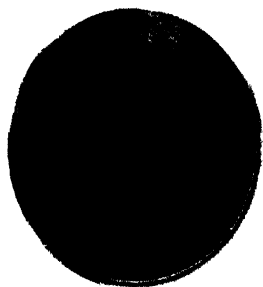


Fig. 1

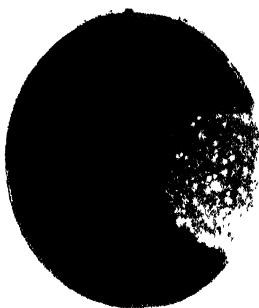


Fig. 3

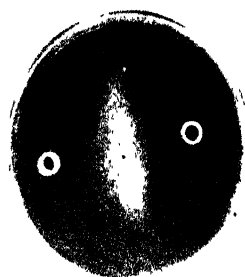
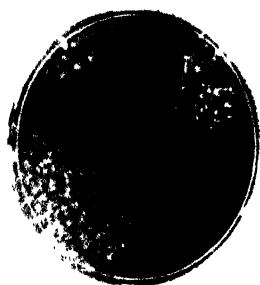


Fig. 5

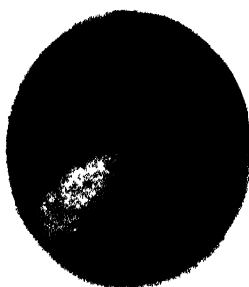


Fig. 6

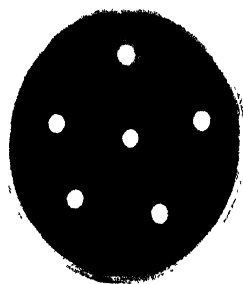


Fig. 8



## The Assimilation of Amino-acids by Bacteria

### 7. The Nature of Resistance to Penicillin in *Staphylococcus aureus*

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**SUMMARY:** Penicillin blocks the assimilation of glutamic acid by *Staphylococcus aureus*; the effective concentration of penicillin is of the same order as that required to inhibit growth of the organism whether the culture is sensitive or resistant to penicillin. Serial subcultivation in increasing concentrations of penicillin results in the selection of resistant mutants; as resistance increases, the ability of the cells to assimilate glutamic acid decreases. The efficiency of the assimilation process in highly resistant cells is poor, but they can synthesize all their amino-acid requirements from ammonia and glucose.

The decrease in assimilatory efficiency as resistance to penicillin increases is correlated with an increase in the ability of the cells to synthesize amino-acids. Reverse mutations, having decreased ability to synthesize certain amino-acids, were obtained from highly resistant strains and had increased sensitivity to penicillin. Strains of *Staph. aureus* requiring several amino-acids as nutrients have been 'trained', by subcultivation in media progressively more deficient in amino-acids, to dispense with the addition to the medium of all amino-acids other than cystine and histidine; the increase in synthetic ability was accompanied by a marked increase in penicillin resistance.

It is suggested that penicillin interferes with the mechanism whereby certain amino-acids are taken into the cell, and that the sensitivity of the cell to penicillin is then determined by the degree to which its growth processes are dependent upon assimilation of preformed amino-acids rather than upon their synthesis.

Previous papers in this series have shown that Gram-positive bacteria are able to assimilate certain amino-acids and to concentrate them in the free state within the cells prior to utilization (Gale, 1947*a*; Taylor, 1947). In parenthesis it may be noted that the word 'assimilation' as used in the present and previous papers of this series denotes transfer from the external environment into the cell, and does not connote incorporation, for example, into protein. The free amino-acids thus accumulated within the cells provide a reservoir which is drawn upon for anabolic processes. The concentration of free amino-acid attained within the cell is determined by the balance between the rate at which the amino-acid enters the cell and the rate at which it is there utilized (Gale & Mitchell, 1947; Gale, 1947*b*). The passage of amino-acids across the cell wall may be by diffusion as in the case of lysine, or it may be by a process requiring energy supplied by some exergonic metabolism. When penicillin is added to a growing culture of *Staph. aureus*, the cells become progressively less able to assimilate glutamic acid until eventually this amino-acid does not enter the cell at all. Since the utilization of glutamic acid within the cell is unaffected by penicillin, the internal reservoir is steadily depleted until protein synthesis ceases (Gale & Taylor, 1947). Penicillin thus prevents the passage of glutamic acid, and probably other amino-acids, into the cell.



If the antibiotic properties of penicillin are due primarily to its capacity to prevent the passage of amino-acids into the cell, it should be possible to correlate this effect with the acquisition of resistance when organisms are subjected to serial subcultivation in increasing concentrations of penicillin.

Demerec (1945) has studied the acquisition of resistance to penicillin under these conditions and has shown that cultures of *Staph. aureus* contain a small number of mutants whose resistance is greater than that of the majority of the cells. When such cultures are subjected to a concentration of penicillin which limits the growth of most of the cells, a selective growth of the resistant mutants takes place. Repetition of the process results in the progressive selection of mutants of steadily increasing resistance. The resistant cells arise by spontaneous mutation and not as a result of the action of penicillin on the bacterial cells; the mutation rate is of the order of 1 in  $10^7$ – $10^8$  generations. A preliminary note (Gale, 1947c) reported that resistant mutants selected by cultivation in penicillin are less efficient in assimilating glutamic acid than the sensitive parent strains. Bellamy & Klimek (1948b) trained a strain of *Staph. aureus* to a resistance 60,000 times that of the parent culture and have selected mutants which are Gram-negative, pleomorphic and strict aerobes. Investigations of the amino-acid metabolism of these highly resistant organisms (Gale & Rodwell, 1948) show that they are able to synthesize all their amino-acid requirements from ammonia and glucose, although their catabolic activities are significantly the same as those of the parent strains.

In this communication we show that the penicillin resistance of *Staph. aureus* is determined by the degree to which its growth processes are independent of the assimilation of preformed amino-acids. Penicillin prevents the passage of such amino-acids across the cell wall, and consequently protein synthesis within the cell is stopped unless the cell is able to synthesize its constituent amino-acids instead of taking them preformed from the external environment.

#### *Organisms and methods*

The greater part of the work described below was carried out with two strains of *Staph. aureus*: (i) *Staph. aureus* 6773: a strain isolated from a nasal swab of a patient treated with penicillin and having a penicillin sensitivity on isolation of 5–9 units/ml.; isolated and given to us by Dr B. Topley; (ii) *Staph. aureus* 209: obtained from the American Type Culture Collection and sent to us by Dr W. D. Bellamy; penicillin sensitivity = 0.05–0.06 units/ml. Other strains of *Staph. aureus* mentioned below were isolated by members of the staff of the Cambridge Pathology Department.

*Preparation of resistant strains.* These were prepared in the usual manner by serial subcultivation in the presence of increasing concentrations of penicillin. The highly resistant organism 209 ( $P_{60}T_{35}$ ) was prepared by Bellamy & Klimek (1948b), who kindly gave us cultures for these experiments. These highly resistant organisms will grow in the presence of 4 mg. crystalline penicillin (c. 7000 units)/ml. medium. Commercial crystalline penicillin was used throughout.

*Investigation of nutrition of organisms.* The nitrogen requirements of staphylococci were investigated by Gladstone (1937); his methods were followed in this work. In general, a complete nutrient medium consisting of salts, nicotinamide, aneurin and pure amino-acids (Gladstone, 1937) was prepared and components then omitted one at a time to determine the effect upon the growth of the organism. Gladstone also showed that staphylococci can be trained to dispense with added amino-acids if these are withdrawn progressively from the medium and their nitrogen equivalent supplied by ammonium ions. The same procedure was used to obtain the non-exacting cultures of *Staph. aureus* 209 and 6773 described later in this paper.

*Investigation of glutamic acid assimilation.* The passage of glutamic acid from the external environment into the cells and its accumulation there as the free amino-acid was investigated as described previously (Gale, 1947*a*; Gale & Mitchell, 1947; Gale & Taylor, 1947). Quantities of glutamic acid are expressed in terms of  $\mu\text{l.}$ ;  $22.4 \mu\text{l.}$  glutamic acid =  $1 \mu\text{mol.}$

### *Preliminary investigations*

#### *Effect of increased resistance on the blocking of glutamic acid assimilation*

The investigations on the effect of penicillin on glutamic acid assimilation (Gale & Taylor, 1947) were carried out with a strain of *Staph. aureus* sensitive to 0.08 unit penicillin/ml. With this organism the assimilation of glutamic acid was completely prevented in 90 min. by the addition of 5 units penicillin/ml. and in 180 min. by 0.1 unit/ml. In order to determine whether the effective concentration of penicillin varied with the resistance of the organism, the experiment was repeated with *Staph. aureus* 6773 trained to a resistance of 60 units/ml. Penicillin was added to cultures after  $3\frac{1}{2}$  hr. growth and the cultures then left in the incubator for a further period of 8 hr.—a period equivalent to that required in former tests for the limiting concentration of penicillin to exert its full effect. The organisms were then harvested and their ability to take up glutamic acid determined as previously described (Gale & Taylor, 1947). In this case the assimilation was unaffected by 1.0 unit penicillin/ml., decreased by 44% in the presence of 10 units/ml., and completely prevented by 100 units/ml. It is clear that an increase in the resistance of the culture as tested by the growth inhibition was accompanied by an increase in the amount of penicillin needed to prevent the passage of glutamic acid into the cell.

#### *Inhibition of internal metabolism*

Previous studies have shown that penicillin interferes with the passage of glutamic acid across the cell wall. This process was studied by investigating alterations in the level of free glutamic acid within the cell. This level is affected by the rate of passage of the amino-acid into the cell and by the rate of its utilization within the cell (Gale & Mitchell, 1947). Consequently if the passage of glutamic acid into the cell is to be studied by this method, it is necessary to inhibit its utilization within the cell. Utilization involves at least two processes: condensation of the amino-acid, with others, into protein (Gale, 1947*b*),

and entrance into the 'metabolic pool' of the cell, without protein formation (Gale & Mitchell, 1947). The protein synthesis takes place only in growing cells and can be eliminated by working with well-washed suspensions of cells. The remaining metabolism (transfer of glutamic acid to the metabolic pool), which takes place in resting and growing cells, can be inhibited by suitable concentrations of certain triphenylmethane dyes. The rates of these two processes vary with the age of the culture from which the cells are harvested. This can be shown (Gale, 1947*b*) by harvesting cells at various times during the growth period and estimating: (1) the internal level of free glutamic acid directly after harvesting; (2) the level attained within the cell after it has been allowed to come into equilibrium, in washed suspension, with an external glutamic acid concentration equal to that in the medium during growth; (3) the level attained within the cell treated as in (2) but in the presence of a concentration of crystal violet which inhibits the transfer of glutamic acid to the metabolic pool. Each level determined represents the balance between the rates of assimilation and utilization under the conditions used. Previous experiments of this nature were carried out with *Streptococcus faecalis* where it was shown that: (a) the rate of protein formation (represented by level 2 – level 1) was greatest during the early stages of growth, fell off during growth and ceased when growth ceased; (b) the rate of the dye-inhibitable metabolism (transfer into the metabolic pool, represented by level 3 – level 2), was greatest during the later stages of growth but fell rapidly after growth had ceased; (c) the rate of assimilation (represented by level 3) was approximately constant throughout the growth period.

In *Strep. faecalis* the rate of dye-inhibitable metabolism may approach half the rate at which the amino-acid enters the cell. When similar experiments were carried out on *Staph. aureus* it was found that the rate of the dye-inhibitable part of the glutamic acid utilization was much smaller, in comparison with the rate of assimilation, in these cells than in *Strep. faecalis*. *Staph. aureus* is about ten times as sensitive to crystal violet as *Strep. faecalis* when judged by the growth inhibition test; investigations on the assimilation of glutamic acid in the presence of crystal violet in experiments similar to those previously described (Gale & Mitchell, 1947) showed that a similar internal inhibition took place with a consequent increase in the level of free glutamic acid attained within the cell, but that optimal effects were obtained with a concentration of dye only 1/50–1/100 of that required for *Strep. faecalis* cells. In the latter the presence of the dye may result in an increase in the internal level of glutamic acid to 300–400% of that attained in the absence of dye; in *Staph. aureus* the increase in level is 5–15% of that attained in the absence of dye.

Fig. 1 shows the curves obtained for internal glutamic acid levels, determined under the three conditions described above, for *Strep. faecalis* and *Staph. aureus*. The picture of glutamic acid utilization differs in the two organisms. In *Staph. aureus* the rate which it is built into protein is nearly equal to the rate of uptake of glutamic acid in young cultures, so that little free glutamic acid accumulates in such cells; this rate decreases as growth continues, so that the amount of free glutamic acid in the cell increases and reaches a maximum level

at the end of growth when protein formation ceases. At all times during the growth phase the rate of the utilization process which is inhibitable by dye, i.e. transfer to the metabolic pool, is small in comparison with the rate of uptake of glutamic acid.

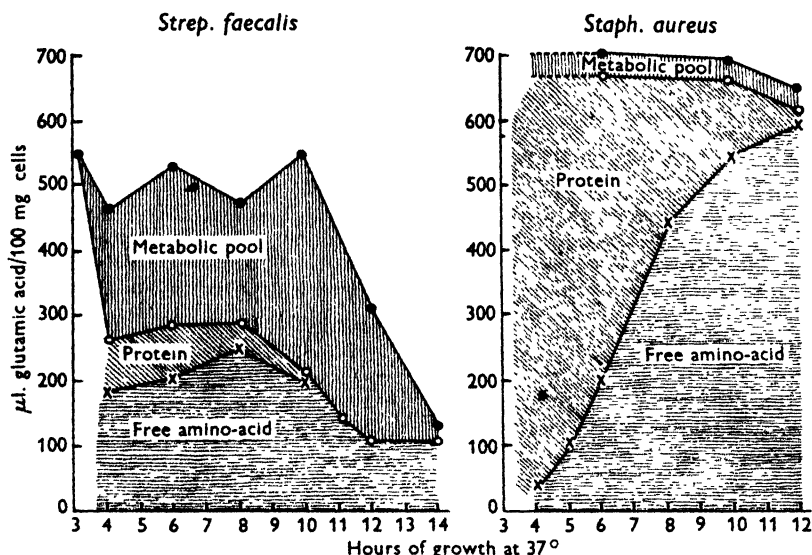


Fig. 1. Utilization of glutamic acid by *Strep. faecalis* and *Staph. aureus*. Curves represent internal level of glutamic acid ( $\mu\text{l.}/100 \text{ mg. cells}$ ) in cells: (1)  $\times$ — $\times$  harvested from growth medium containing  $200 \mu\text{l.}$  glutamic acid/ml. (2)  $\circ$ — $\circ$  incubated for 1 hr. at  $37^\circ$  in washed suspensions in salt solution containing 1% glucose and  $200 \mu\text{l.}$  glutamic acid/ml. (3)  $\bullet$ — $\bullet$  incubated for 1 hr. at  $37^\circ$  in washed suspension in salt solution containing 1% glucose,  $200 \mu\text{l.}$  glutamic acid/ml. and crystal violet at a final concentration  $1/10^4$  for *Strep. faecalis* and  $1/10^6$  for *Staph. aureus*. Rate of 'condensation' of glutamic acid into protein in comparison with rate of assimilation is given by (2-1). Rate of entry of glutamic acid into metabolic pool, in comparison with rate of assimilation, given by (3-2).

In order, therefore, to study the process whereby glutamic acid passes across the cell wall, preparations of cells were made by harvesting cells from 6-7 hr. cultures, washing them in water and then treating the washed cells, in a suspension containing *c.* 1-2 mg. dry weight of cells/ml., with crystal violet in final dilution  $1/10^6$ . In such cells glutamic acid enters the cell until a steady state is obtained, and the level attained within the cell is determined by the ability of the cell to maintain a difference in concentration across the cell wall and is not affected by internal metabolic processes.

### The assimilation process in cells of differing resistance to penicillin

#### Concentration gradient across cell wall

Taylor (1947) showed that the ability to concentrate glutamic acid inside the cells differs in different micro-organisms. All the Gram-positive bacteria examined have exhibited this ability, and, of the species studied, strains of *Staph. aureus* effected the highest internal concentration for a given external

concentration. It was thought that the sensitivity to penicillin might be correlated with this ability to concentrate glutamic acid across the cell wall. To test this, a number of bacteria of differing penicillin sensitivities were grown, and washed suspensions of cells treated with crystal violet were prepared as described above. These cells were then incubated for 1 hr. at 37° in a suitable buffer mixture containing 200  $\mu$ l. glutamic acid/ml. and 1 % glucose as energy source; after incubation the internal level of free glutamic acid was determined as previously described (Gale, 1947a). Table 1 shows

Table 1. *Penicillin resistance and internal concentration of glutamic acid*

Organisms grown for 6 hr. in medium B (Gale, 1947a) harvested and washed in water; suspended for 1 hr. at 37° at a suspension strength of approx. 1 mg. dry weight of cells/ml. in buffer-salt solution containing 200  $\mu$ l. glutamic acid/ml., 1 % glucose and crystal violet to a final dilution of 1/1,000,000 for *Staph. aureus* and *B. subtilis* or 1/10,000 for *Strep. faecalis*; cells then washed in water and internal glutamate concentration assayed.

| Organism               | Strain                                 | Growth inhibition test<br>(units penicillin/ml.) |           | Internal<br>concentration of<br>glutamic acid<br>( $\mu$ l./100 mg. cells) |
|------------------------|--|--|-----------|--|
|                        |  | Growth   | No growth |  |
| <i>Staph. aureus</i>   | 563                                    | 0.02   | 0.04      | 1165   |
| <i>B. subtilis</i>     | St.                                    | 0.04   | 0.06      | 26   |
| <i>Staph. aureus</i>   | 209                                    | 0.05   | 0.08      | 560  |
| <i>Staph. aureus</i>   | D                                      | 0.06   | 0.08      | 660  |
| <i>Strep. faecalis</i> | ST                                     | 6.0  | 8.0       | 534  |
| <i>Staph. aureus</i>   | 6773                                   | 5.0  | 10.0      | 880  |
| <i>Staph. aureus</i>   | 6773                                   | 15   | 20        | 880  |
| <i>Staph. aureus</i>   | 6773                                   | 60   | 70        | 750  |
| <i>Staph. aureus</i>   | 6773                                   | 250  | 300       | 740  |
| <i>Staph. aureus</i>   | 6773                                   | 2000   | 4000      | 705  |
| <i>Staph. aureus</i>   | 6773                                   | 6000   | —         | 0  |
| <i>Staph. aureus</i>   | 209 (P <sub>80</sub> T <sub>35</sub> ) | 7000   | —         | 0  |

that there is no correlation between the internal levels attained and the penicillin sensitivity as determined in the growth inhibition test. The organism 6773 was trained to a final resistance of 6000 units penicillin/ml. and tested at various stages during the training process. It can be seen that the internal level does decrease slowly as resistance increases, but this decrease bears little relation to the increase in penicillin resistance. It will be seen below that the ability to assimilate glutamic acid decreases as resistance increases and that the falling internal levels shown in Table 1 may be a reflexion of the fact that the internal environment is not saturated in the cells of high resistance. At the highest penicillin level (6000 units/ml.) the cells became Gram-negative, and it was no longer possible to determine any free glutamic acid within the cells. These results accord with the findings of Taylor (1947) that free amino-acids are not concentrated within Gram-negative cells.

#### *Dependence of internal concentration on external concentration*

In earlier studies on the assimilation of glutamic acid by *Strep. faecalis*, curves were given showing the dependence of the internal concentration on the external concentration (Gale, 1947a). These curves were, however, determined

with washed cell suspensions, and the internal levels found were affected by internal metabolic processes. When the curves are redetermined using dye-treated washed cells, they then assume the shape shown in Fig. 2. The internal concentration of glutamic acid is independent of the external concentration except for very low values of the latter. Fig. 2 shows curves obtained for *Strep. faecalis* (cf. Gale, 1947a, Fig. 8) and for *Staph. aureus*. The values attained at saturation are different in the two species of cell, but it is improbable, as shown above, that this is a factor in determining penicillin sensitivity.

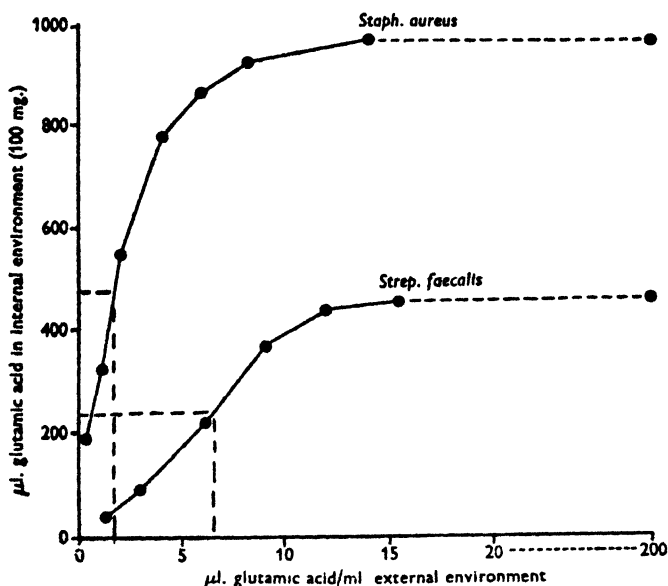


Fig. 2. Relation between internal and external glutamate concentration. Cells harvested from deficient medium, washed and treated with crystal violet to inhibit internal metabolism, incubated for 1 hr. in presence of 1% glucose and glutamic acid at concentrations shown, and internal level of free glutamic acid then assayed.

Further, the slope of the curve relating internal concentration to external concentration for low values of the latter is steeper for the *Staph. aureus* than for the *Strep. faecalis* strain used. This means that *Staph. aureus* effected a more efficient concentration of glutamic acid at low external concentrations than *Strep. faecalis*. The *Staph. aureus* strain used is sensitive to 0.08 unit penicillin/ml., while the *Strep. faecalis* strain is resistant to 5 units penicillin/ml. As a measure of the slope of the concentration curve, we have defined the 'assimilation constant' as that external concentration which gives rise to an internal concentration equal to half that attained at saturation. The reciprocal of the assimilation constant is thus a measure of the ability of the cell to assimilate glutamic acid.

#### Variation of 'assimilation constant' with penicillin resistance

Fig. 3 shows the internal concentration curves, expressed as percentage internal saturation, for organisms of differing penicillin sensitivity. The organisms were grown as usual for 6 hr., harvested, washed, treated with

crystal-violet and the usual suspensions made. The internal level of glutamic acid was determined over a range of external concentrations. When the external concentration is small, assimilation may result in a lowering of the external concentration; to avoid significant alterations of this nature, the cells were suspended in large volumes of solution such that the suspension contained 0.2–0.5 mg. dry weight bacteria/ml.

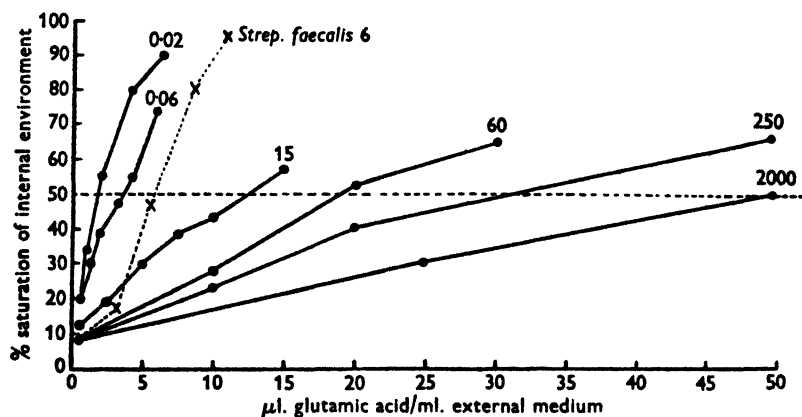


Fig. 8. Variation of assimilation constant with penicillin resistance. Penicillin resistance as determined by growth inhibition test is shown in units/ml. at the top of each curve. All curves—except the broken one—refer to *Staph. aureus* strains.

Determinations were first carried out with four strains of *Staph. aureus* of differing penicillin resistances. Fig. 3 shows that the value of the assimilation constant increases with the penicillin resistance. In order to determine whether this was a true correlation, the organism 6773 of resistance 15 units penicillin/ml. was trained by the usual method to an ultimate resistance of 6000 units/ml., and the assimilation constant was determined on cells having levels of resistance of 15, 60, 250, 2000 and 4000 units/ml. respectively. Fig. 3 shows that the assimilation constant increased markedly as the resistance increased. Fig. 4 shows the correlation between the value of the assimilation constant and the log of the penicillin resistance for the various organisms tested. Most of the points were determined with *Staph. aureus*, but values obtained for a sensitive *Bacillus subtilis* and a moderately resistant *Strep. faecalis* appear to fall on the curve obtained with the staphylococci. It is clear that the assimilation constant increases rapidly with penicillin resistance. Consequently the ability of the cell to assimilate glutamic acid decreases as the resistance increases; serial subcultivation in penicillin selects resistant cells from the culture, and these cells have less efficient assimilatory mechanisms than the sensitive parent cells.

#### *Nature of the highly resistant cells*

When the resistance of *Staph. aureus* 6773 is increased from 2000 to 6000 units/ml., the cells undergo changes in morphological, cultural and staining characteristics as described by Bellamy & Klimek (1948*b*) for *Staph. aureus* 209.

The resistant organisms obtained from both strains are highly pleomorphic, strictly aerobic and Gram-negative (see Pl. 1). The general properties and amino-acid metabolism of these organisms have been described by Bellamy & Klimek (1948*b*) and by Gale & Rodwell (1948), respectively. The amino-acid

● 76

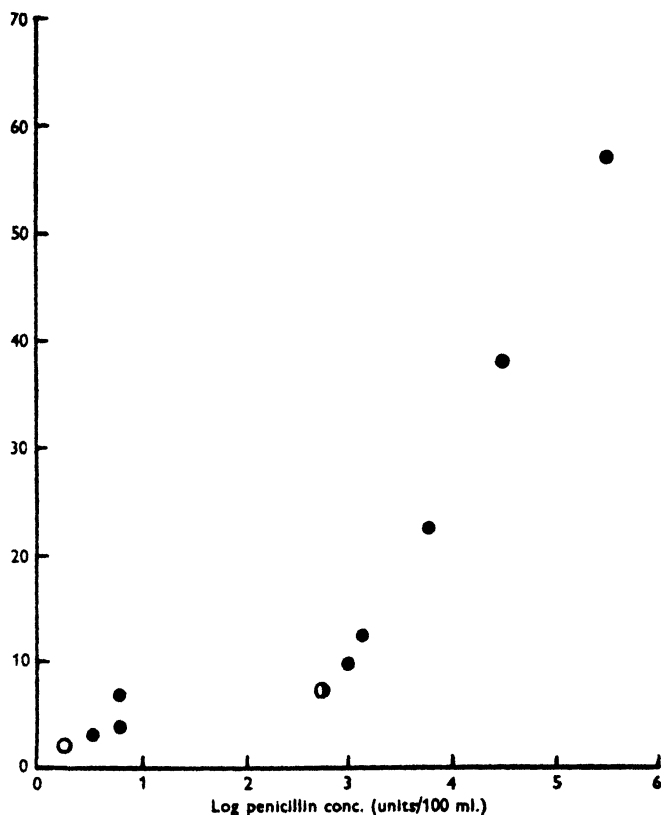


Fig. 4. Relation between value of assimilation constant and log of penicillin resistance expressed in units/100 ml. ● *Staph. aureus*. ○ *B. subtilis*. ◐ *Strep. faecalis*. (Ordinate = assimilation constant expressed in μl. glutamic acid/ml.).

breakdown by the two resistant organisms is significantly the same as that accomplished by the parent strains; all four organisms possess arginine dihydrolase and urease to approximately the same degree of activity.

#### *Relation between amino-acid synthesis and penicillin resistance*

#### *Synthetic abilities of Staphylococcus aureus mutants obtained by penicillin-training procedures*

The nutritional requirements of the various strains of *Staph. aureus* obtained in this work were studied by the method of Gladstone (1937). Gale & Rodwell (1948) found that, while the parent penicillin-sensitive strains of *Staph. aureus*



6773 and 209 were exacting towards nicotinamide, aneurin and a range of amino-acids, the highly resistant organisms obtained therefrom could grow in a medium containing salts, ammonium ions, glucose and aneurin.

Table 2. *Relation between synthetic abilities and penicillin resistance in Staphylococcus aureus*

Mutants were selected by training in the presence of penicillin.

Nutritional requirements investigated as described by Gladstone (1937)

+ = presence essential for growth in same time as in complete medium; no growth takes place in absence.

— = presence not essential for growth in same time as in complete medium.

(88) etc. = in absence of the given amino-acid, growth equal to that in complete medium took place after a delay of 88 etc. hr.

| Organism               | ... | 6773 | 6773 | 6773 | 209  | 209  | 209               | 209  |
|------------------------|-----|------|------|------|------|------|-------------------|------|
|                        |     |      |      |      |      |      | (reverse mutants) |      |
| Gram reaction          | ... | +    | +    | —    | +    | —    | +                 | +    |
| Penicillin resistance: |     |      |      |      |      |      |                   |      |
| after 36 hr.           | ... | 5    | 2000 | 6000 | 0.05 | 7000 | 400               | 1000 |
| after 88 hr.           | ... | 9    | 2000 | 6000 | 0.05 | 7000 | 8000              | 1700 |

Nutrients:

|               |      |      |   |   |   |       |      |
|---------------|------|------|---|---|---|-------|------|
| Nicotinamide  | +    | +    | — | + | — | —     | —    |
| Aneurin       | +    | +    | + | + | + | +     | +    |
| Proline       | (88) | (88) | — | + | — | (88)  | (64) |
| Histidine     | +    | +    | — | + | — | (64)  | (64) |
| Valine        | +    | +    | — | + | — | (88)  | (88) |
| Glycine       | +    | (88) | — | + | — | (160) | +    |
| Glutamic acid | (40) | —    | — | — | — | (64)  | —    |
| Aspartic acid | +    | (64) | — | + | — | (160) | (64) |
| Leucine       | (40) | (40) | — | + | — | —     | —    |
| Cystine       | +    | +    | — | + | — | +     | +    |
| Phenylalanine | (40) | (64) | — | — | — | —     | —    |
| Arginine      | +    | +    | — | — | — | +     | +    |

Table 2 shows the nutritional requirements of these organisms and of other mutants obtained therefrom. The organism selected from *Staph. aureus* 6773 at a resistance level of 2000 units/ml. is a pigmented, Gram-positive *Staph. aureus* with nutritional requirements intermediate between those of the sensitive parent strain and those of the highly resistant non-exacting organism. Thus the organism resistant to 2000 units is completely independent of pre-formed glutamic acid and will adapt to the absence of glycine and aspartic acid in 88 and 64 hr. respectively, while the parent organism cannot dispense with either glycine or aspartic acid and adapts to the absence of glutamic acid in 40 hr.

Table 2 also sets out the nutritional requirements of two organisms obtained by reverse mutation from the highly resistant 209 ( $P_{60}T_{35}$ ). These organisms were isolated by the procedure described by Bellamy & Klimek (1948b) and are pigmented Gram-positive *Staph. aureus*. When tested for penicillin resistance by the usual method involving incubation for 36 hr. the two organisms are resistant to 400 and 1000 units/ml. respectively. Likewise their nutritional requirements are wide on initial incubation. On continued incubation, how-

ever, they adapt to the absence of several amino-acids: presumably the experimental procedure results in the selection of less exacting mutants (i.e. better amino-acid synthesizers). On initial test both organisms required proline, histidine, valine, aspartic acid, cystine, glycine and arginine while the organism resistant to 400 units/ml. also required glutamic acid. After 88 hr. incubation growth of the same organism occurred in the absence of proline, histidine, valine and glutamic acid, whereas the 1000-unit organism no longer required preformed proline, histidine, valine and aspartic acid. At the same time, the resistance of both organisms had approximately doubled.

The highly resistant 209(P<sub>60</sub>T<sub>36</sub>) organism can synthesize nicotinamide, and this ability is retained by the reverse (i.e. amino-acid requiring) mutants. It is interesting that whereas the initial parent 209 does not require added arginine, both reverse mutants tested have lost the power of synthesizing arginine.

It appears from the data in Table 2 that the penicillin-resistant organisms have high synthetic abilities; that penicillin training results in the selection of synthetically competent cells; and that reverse mutation to a more sensitive form is accompanied by loss of synthetic ability, the sensitivity of the reverse mutants depending upon the degree of synthetic competence. These results correlate well with the previous findings that increase in penicillin resistance is accompanied by decreased efficiency in amino-acid assimilation.

#### *Penicillin resistance of non-exacting mutants of Staphylococcus aureus*

Gladstone (1937) showed that nutritionally non-exacting strains of *Staph. aureus* can be obtained from exacting strains when the organisms are serially subcultivated in medium from which essential amino-acids are progressively withdrawn. Using the technique described by Gladstone we have endeavoured to train the strains 6773 and 209 to synthesize their amino-acid requirements from ammonia and glucose. It has been possible to train both strains to dispense with the addition of all amino-acids other than cysteine and histidine. Gladstone recorded that some strains can dispense with cysteine if provided with thiolacetic acid, but we have not been able to accomplish this change with the two organisms under investigation. The penicillin resistance of the comparatively non-exacting cultures was determined as usual and compared with that of the exacting parent cultures. Table 3 shows that the non-exacting mutants selected by the nutritional restriction procedure have much higher resistance than the parent strains; in the case of *Staph. aureus* 209, the parent strain requires seven amino-acids and is sensitive to 0.05–0.06 unit penicillin/ml., while the mutant, which requires only cysteine and histidine as amino-acid nutrients, is resistant to 250 units/ml.

#### DISCUSSION

The early studies on assimilation of glutamic acid by *Staph. aureus* showed that the addition of penicillin to the medium during growth was followed by an impairment of the assimilation process which eventually led to a complete cessation of the passage of the amino-acid into the cell (Gale & Taylor, 1947).

The amino-acid which accumulates within the cell acts as a reservoir which is drawn upon for anabolic processes, so that, when the entry of the amino-acid is prevented, the reservoir is depleted and anabolic processes cease. When the culture is serially subcultivated in increasing concentrations of penicillin, resistant mutants are selected, and the higher the resistance of these mutants, the less efficient is their assimilation of glutamic acid. Growth of a cell involves synthesis of protein and consequently the provision of its constituent amino-

Table 3. *Relation between synthetic abilities and penicillin-resistance in Staphylococcus aureus*

| Mutants obtained by training in depleted media (Gladstone, 1937) |     |      |      |      |     |
|--|-----|------|------|------|-----|
| Organism   | ... | 6773 | 6773 | 209  | 209 |
| Penicillin resistance  |     | 5    | 100  | 0.06 | 250 |
| (units/ml.) 36 hr. test  |     |      |      |      |     |
| Nutrients required:  |     |      |      |      |     |
| Nicotinamide   |     | +    | +    | +    | +   |
| Aneurin  |     | +    | +    | +    | +   |
| Proline  |     | +    | —    | +    | —   |
| Histidine  |     | +    | +    | +    | +   |
| Valine   |     | +    | —    | +    | —   |
| Glycine  |     | +    | —    | +    | —   |
| Glutamic acid  |     | +    | —    | —    | —   |
| Aspartic acid  |     | +    | —    | +    | —   |
| Leucine  |     | +    | —    | +    | —   |
| Cystine  |     | +    | +    | +    | +   |
| Arginine   |     | +    | —    | —    | —   |

acids; such provision can be made either by synthesis or by assimilation of preformed amino-acids from the external environment. If a cell loses the ability to synthesize a given amino-acid, then the growth of that cell becomes dependent upon its ability to assimilate that amino-acid preformed. The resistant mutants are less able to assimilate glutamic acid than the sensitive ones, and cells selected for high levels of penicillin resistance are those with small assimilation efficiency. If these cells are to grow, they must be able to synthesize the amino-acids which they are unable to take from the environment; if the penicillin resistance is pushed to its limit, then it follows that the cells then selected must be able to synthesize all those amino-acids whose assimilation is prevented by penicillin.

The assimilation studies have been restricted to glutamic acid, since this is the only amino-acid which can be effectively studied by the present technique (Gale, 1947*a*). It has always been considered possible that the effects studied were symptomatic of general changes in assimilatory processes rather than specific for glutamic acid. The results given in the latter part of this paper support this. The increase in penicillin resistance is correlated with a decrease in the ability to assimilate glutamic acid, but when we examine the synthetic abilities which enable the organism to overcome the assimilation impairment, we find that there is a general gain in the ability to synthesize amino-acids, while glutamic acid itself appears to be comparatively unimportant. For

example, glutamic acid is not an essential nutrient for *Staph. aureus* 209 at any stage. Glutamic acid is taken into the cell by an active process requiring energy, and the information at our disposal suggests that many other amino-acids are assimilated in a like manner, e.g. aspartic acid and histidine (Gale, 1947*a*). The passage of lysine into the cell is a physical process not requiring a concomitant energy-yielding process, and may be exceptional; the assimilation of lysine is not prevented by penicillin (Gale & Taylor, 1947), and lysine is not an essential nutrient for the organisms studied here.

It seems probable that penicillin acts by preventing the passage across the cell wall of those amino-acids whose migration involves an active process. Resistant mutants are those whose growth is less dependent upon this kind of assimilation and whose ability to make protein escapes from the restriction imposed by penicillin, by synthesizing amino-acids instead of assimilating them preformed from the environment. Knight (1936) and Lwoff (1943) have both put forward the hypothesis that organisms become nutritionally exacting as a result of prolonged growth in media rich in the essential growth factors and amino-acids; growth is supposed to proceed more effectively by the assimilation of preformed protoplasmic components rather than by synthesis, and in due course, the synthetic abilities are lost by disuse. The action of penicillin is to reverse this process.

The facts presented in this communication have been obtained with mutants of *Staph. aureus*, and it is important to determine whether the results are applicable to other organisms, to explain differences in penicillin resistance between genera and species. Table 4 presents data, gathered from the literature, concerning the amino-acid requirements and penicillin sensitivity of a variety of organisms. It can be seen that there is general agreement with the hypothesis that penicillin sensitivity can be correlated with the dependence of the growth process on preformed amino-acid assimilation, i.e. that the more synthetically competent the organism, the greater its resistance to penicillin. The assimilation studies so far described have been carried out with Gram-positive organisms, since the ability of these organisms to concentrate amino-acids within the cell has provided the point of experimental attack.

The nutritional requirements of certain Gram-negative organisms indicate that these must also be able to assimilate some preformed amino-acids, although such assimilation is not accompanied by an internal concentration of the free amino-acid prior to further utilization. The Gram-positive group of bacteria is, in general, highly exacting towards amino-acids and sensitive to penicillin; but within the Gram-negative group we also find degrees of disability in amino-acid synthesis, and there is again a correlation between the degree of synthetic disability and sensitivity to penicillin.

The question arises, to what extent is this prevention of assimilation processes the primary point of action of penicillin? Penicillin only kills cells that are growing in its presence; the assimilation process is blocked only when the cell grows in its presence. This suggests that the primary action of penicillin is to inhibit the formation of a substance whose synthesis is essential for the assimilation process to occur. If nutritionally exacting cells are to grow—in

Table 4. Nutritional requirements and penicillin resistance of certain bacteria: collected data

|  | <i>Escherichia coli</i> | <i>Shig. dysenteriae</i> | <i>Proteus vulgaris</i> | <i>Eberthella typhosa</i> | <i>Strep. faecalis</i> | <i>Lacto-bacillus casei</i> | <i>C. diph-theriae</i> | <i>Staph. aureus</i> | <i>Bacillus anthracis</i> | <i>Strep. haemolyticus</i> | <i>Neisseria intracellularis</i> | <i>Neisseria gonorrhoeae</i> |
|--|-------------------------|--------------------------|-------------------------|---------------------------|------------------------|-----------------------------|------------------------|----------------------|---------------------------|----------------------------|----------------------------------|------------------------------|
| Gram reaction  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | -                                | -                            |
| Penicillin sensitivity range in units/ml. (Duguid, 1946) ... | 80-300                  | 30-100                   | 3-30                    | 10-30                     | 1-10                   | 0.1-1                       | 0.03-0.1               | 0.01-0.1             | 0.01-0.1                  | 0.01-0.03                  | 0.003-0.03                       | 0.003-0.01                   |
| Glycine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Alanine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Serine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Cystine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Phenylalanine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Tyrosine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Tryptophan   | -                       | -                        | -                       | +                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Threonine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Valine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Leucine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Isoleucine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Glutamic acid  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Aspartic acid  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Histidine  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Lysine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Arginine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Methionine   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Proline  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Hydroxyproline   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Nicotinic acid   | -                       | +                        | +                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Aneurin  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Riboflavin   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Pantothenate   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Pyridoxal  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Biotin   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Folic acid   | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Purines, etc.  | -                       | -                        | -                       | -                         | +                      | +                           | +                      | +                    | +                         | +                          | +                                | +                            |
| Nutrition references:  | 1, 2                    | 3                        | 4                       | 5                         | 5                      | 5                           | 6                      | 7, 8                 | 9                         | 10                         | 11, 12                           | 13, 14                       |

(See References)

the absence of mutational changes—they must synthesize this mechanism, necessary for the assimilation of preformed amino-acids, as new cells are formed. Inhibition of the synthesis of a part of this mechanism would impair the assimilation process, and the sensitivity of the cell to penicillin would then be determined by the dependence of its growth processes on the assimilation mechanism. Present indications are that the assimilation mechanism involves ribonucleic acid synthesis which is the primary point of penicillin attack. It is hoped to deal with this point in a later publication.

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#### EXPLANATION OF PLATE

Fig. 1. *Staph. aureus* 6773, parent strain; Gram stain; photographed through green filter. Magnification  $\times 4800$ .

Fig. 2. Highly resistant organism derived from *Staph. aureus* 6773; Gram stain; photographed through green filter. Magnification  $\times 4800$ .

Photomicrographs by V. C. Norfield, Strangeways Research Laboratory, Cambridge.

(Received 15 June 1948)

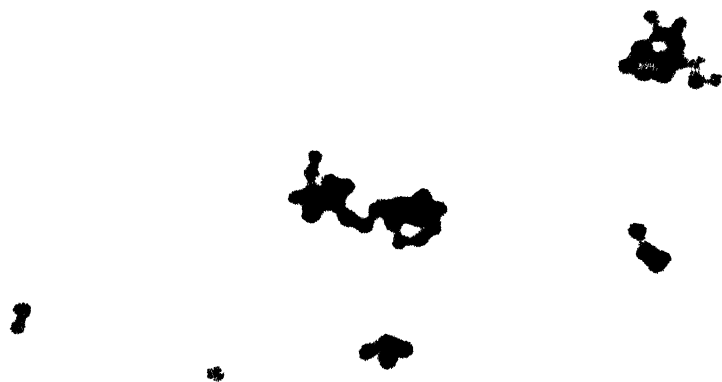


Fig. 1

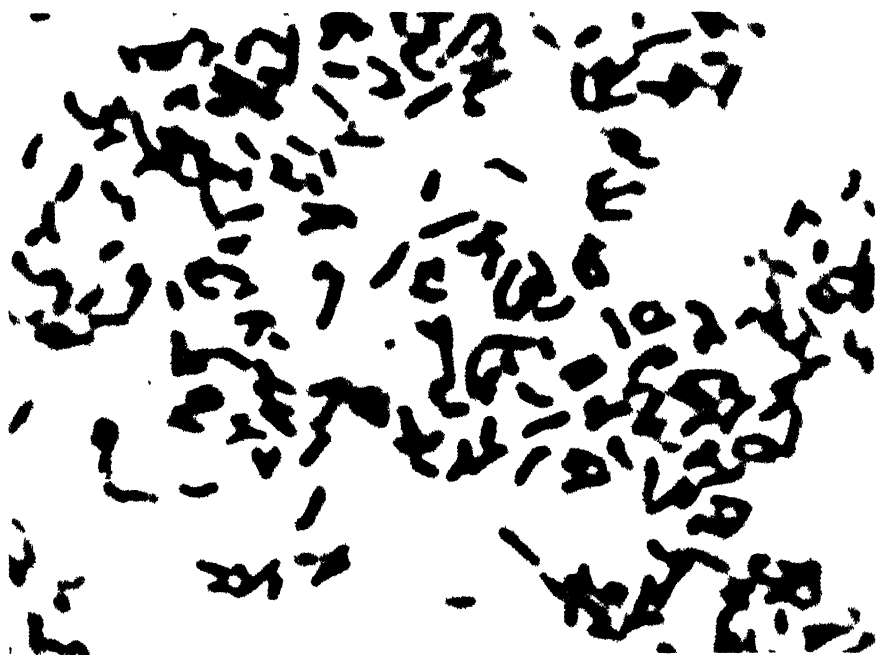


Fig. 2





## The Utilization of Pyridine by Micro-organisms

By F. W. MOORE

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**SUMMARY:** Soil-enrichment cultures in a medium containing 0.1 % (v/v) pyridine as the sole organic compound gave plate counts of more than 1000 million organisms/ml. Organisms of the genus *Proactinomyces* were able to utilize pyridine, aniline, nicotinic acid, nitrobenzene, or phenol + ammonium ion as sole source of carbon, nitrogen and energy.

Very little is known of the decomposition by micro-organisms of simple cyclic compounds. The fact that such compounds do not accumulate in the soil indicates that degradation must occur. The chemical stability of such elementary six-membered ring compounds as benzene and pyridine is well known, and it is unlikely that any decomposition takes place in the soil as a result of purely chemical action. The occurrence of the pyridine ring in the form of nicotinic acid or its derivatives appears to be universal in living cells. Co-enzymes I and II each contain the nicotinamide moiety as an essential part of their molecules. The quantities of pyridine ring compounds which are returned to the soil on the death of animal and vegetable cells are extremely difficult to estimate, but it can be assumed that there is a steady supply of such compounds available for decomposition.

References to the dissimilation of pyridine by micro-organisms are few. Buddin (1914), when attempting to sterilize soil by the use of a pyridine solution, found that the pyridine was rapidly broken down by micro-organisms. Funchess (1917) showed that nitrification, using pyridine, occurred in soil; pyridine was almost as good a source of nitrogen for corn and oats as sodium nitrate. Robbins (1917) demonstrated that the bacterial content of soil rose rapidly after the addition of 0.1 % pyridine. He isolated a species of bacterium which was capable of growing in a medium containing pyridine as the sole source of nitrogen. den Dooren de Jong (1926) found that pyridine could be used as the sole source of nitrogen by *Aerobacter aerogenes*, *Serratia marcescens* and *Bacterium herbicola*. Ostroff & Henry (1939) reported the utilization of pyridine as a source of nitrogen by three species of marine bacteria. Koser & Baird (1944) studied the growth of bacteria on a medium which included nicotinic acid or closely related compounds. They found organisms of the *Pseudomonas fluorescens* group capable of using nicotinic acid as their sole source of carbon, nitrogen and energy. *Serratia marcescens* and related species were able to grow on a similar medium having  $(\text{NH}_4)_2\text{HPO}_4$  as the nitrogen source. None of these organisms was able to grow when pyridine replaced the nicotinic acid.

### *Experimental methods*

**Medium.** The basal culture medium used for most of the work was a modification of that used by Gray & Thornton (1928):  $\text{K}_2\text{HPO}_4$ , 1.0 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g.; NaCl, 0.1 g.;  $\text{CaCl}_2$ , 0.1 g.;  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{KNO}_3$ , 1.0 g.; pyridine A.R.,

1.0 ml.; dist. water to 1000 ml. The pH was adjusted to 7.2–7.4, and the medium sterilized by autoclaving for 20 min. at 15 lb./sq.in. pressure. Solid medium was prepared by adding 400 ml. of 5 % agar (well-washed) to 600 ml. of the basal medium. Occasionally the composition of the medium was modified by the omission of the inorganic source of nitrogen, or by varying the percentage of pyridine.

### *Preliminary enrichment*

Five g. of garden soil were shaken with 50 ml. of quarter-strength Ringer's solution. After standing for 5 min., 1 ml. of this suspension was added to 100 ml. of the basal medium, which included ammonium sulphate, and another 1 ml. was added to 100 ml. of the basal medium containing potassium nitrate. These liquid cultures were incubated at room temperature (15–20°) for 7 days.

Examination of stained and unstained preparations showed large numbers of non-motile, much-branched filamentous organisms, and smaller numbers of motile rods, non-motile cocci and Protozoa. These mixed cultures were subcultured by transferring one loopful of the liquid to 20 ml. of basal medium containing either  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{KNO}_3$ . This subcultivation and enrichment was carried out four times, the cultures being incubated at room temperature for 5 days.

### *Isolation*

Decimal dilutions to 1/10,000,000 of the final enrichment cultures were prepared in sterile quarter-strength Ringer's solution. Two drops (c. 0.1 ml.) of each dilution were spread over the surface of previously poured and surface dried plates of Lemco nutrient agar, which were incubated at room temperature for 10 days. Plates prepared from the highest dilutions (1/10,000,000) averaged 15–20 colonies/plate, indicating that the final enrichment cultures contained at least 1500 million organisms/ml. Isolated colonies were subcultured on Lemco agar slopes, and the resulting growths were again subcultured into the basal pyridine medium which included the same inorganic source of nitrogen as the enrichment culture from which the organism was isolated. Two more subcultures into the pyridine medium were carried out at 5-day intervals to minimize the carry-over of organic matter from the Lemco agar. Tubes showing visible growth were then subcultured into the basal medium without the inorganic nitrogen.

### *Identification*

The organisms forming colonies on Lemco agar plates were classified by microscopical and cultural examination, as follows: *Proactinomyces*, c. 40 %; Gram-negative rods (mainly *Pseudomonas* sp.), c. 30 %; *Actinomyces*, c. 15 %; Gram-positive cocci and rods, c. 15 %.

When these organisms were inoculated in pure culture into the pyridine medium, almost every organism showed some growth in the first subculture. This was probably due to the fact that the initial inoculum was heavy and that a small quantity of organic matter was carried over. It was found that all the

*Proactinomyces* organisms were capable of continued growth in the basal pyridine medium, whereas none of the other organisms would grow in pure culture in it, even when an inorganic nitrogen compound was also present. The large number of organisms of the *Pseudomonas* type found in the final enrichment cultures suggests that, once the pyridine ring is broken by the *Proactinomyces* species, the pseudomonads are able to utilize the decomposition products as sources of carbon, nitrogen and energy.

There were two distinct groups of *Proactinomyces* present. The first group, which included about ten morphologically distinct types, was characterized by the production of a small, rather unstable, branched mycelium, which appeared

Table 1. *Effect of pyridine concentration on the growth of Proactinomyces*

The results were classified by visual examination into four degrees of turbidity: — no visible growth; + slight growth; ++ medium growth; +++ heavy growth.

| Concentration<br>of pyridine<br>% (v/v) | Duration of incubation (days) |    |     |     |     |     |     |
|---|-------------------------------|----|-----|-----|-----|-----|-----|
|   | 2                             | 3  | 5   | 10  | 15  | 24  | 43  |
|   | Relative turbidity            |    |     |     |     |     |     |
| 0.05                                    | +                             | +  | ++  | ++  | ++  | ++  | ++  |
| 0.10                                    | +                             | ++ | +++ | +++ | +++ | +++ | +++ |
| 0.15                                    | +                             | ++ | +++ | +++ | +++ | +++ | +++ |
| 0.20                                    | —                             | +  | ++  | +++ | +++ | +++ | +++ |
| 0.25                                    | —                             | +  | +   | ++  | +++ | +++ | +++ |
| 0.30                                    | —                             | —  | +   | ++  | +++ | +++ | +++ |
| 0.35                                    | —                             | —  | —   | +   | ++  | +++ | +++ |
| 0.40                                    | —                             | —  | —   | —   | +   | ++  | +++ |
| 0.45                                    | —                             | —  | —   | —   | +   | +   | ++  |
| 0.50                                    | —                             | —  | —   | —   | —   | +   | +   |

granular when stained by Gram's method. Surface colonies on Lemco agar were pinkish, glistening and easily emulsified. A heavy sediment was produced in liquid culture. The second group, the various strains of which closely resembled one another, produced a relatively stable, much-branched and matted mycelium. Surface colonies were white, dull, waxy and difficult to emulsify. In liquid culture, a pellicle of the 'powdered chalk' type was produced, together with a flocculent growth which occasionally adhered to, and formed colonies on, the inner surface of the tube.

#### *Growth in various concentrations of pyridine*

The basal medium was modified by omitting the inorganic source of nitrogen, and by adding pyridine to give the following percentage (v/v) concentrations: 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50. All the strains of organisms utilizing pyridine were subcultured from 4-day cultures in 0.10 % (v/v) pyridine medium into the liquid media of varied pyridine concentration. The results are shown in Table 1. All strains seemed to follow the same pattern, growth being more rapid in the lower concentrations and reaching a maximum after 5 days. As the concentration of pyridine was increased, the initial lag

period was extended. In the case of 0.50 % pyridine, no growth was visible for 21 days, and a maximum was not reached until 70–90 days.

The remainder of the investigation was carried out on two strains of *Proactinomyces*, one from each of the two groups mentioned earlier. The basal medium was modified by the substitution of other cyclic compounds in the place of pyridine. The concentrations used were 0.10 % (w/v) of phenol and of nicotinic acid, and 0.10 % (v/v) of aniline and of nitrobenzene. Both organisms were able to use the cyclic compound as the sole source of carbon, nitrogen and energy for their growth, except in the case of phenol, which was utilized when 0.1 % ammonium sulphate was added.

#### *Some products of metabolism*

Crystals formed in liquid cultures of each type of organism; these crystals gave positive reactions for calcium and oxalate. As the carbon:nitrogen ratio of pyridine is approximately 4:1, it is unlikely that the organisms assimilate all the nitrogen present in the medium. Microchemical tests for nitrate, nitrite and hydroxylamine gave uniformly negative results at all stages of growth. The progressive production of ammonia was demonstrated by the use of Nessler's reagent.

#### DISCUSSION

Previous evidence of the growth of pure cultures of micro-organisms in media containing unsubstituted six-membered ring compounds as their sole source of carbon and energy has been provided by Tausson (1929), who demonstrated that benzene was utilized by an organism which he called *Bact. benzoli*. Grant & ZoBell (1944), using absorption of oxygen as evidence of growth, reported that certain marine bacteria could utilize benzene.

The stability of unsubstituted aromatic compounds to microbial attack has been noted by many workers. ZoBell (1948) states that 'aromatic and naphthenic hydrocarbons having side-chains appear to be attacked more readily than similar homologues without side-chains'. Stanier (1948) found that benzene, toluene and xylene were not decomposed by fluorescent pseudomonads. The bacteria studied by Tausz & Peter (1919) failed to attack benzene.

On the other hand, reports of the breakdown of substituted cyclic compounds such as phenol, benzoic acid and nicotinic acid are more numerous (see Gray & Thornton, 1928; Stanier, 1948; and Koser & Baird, 1944). It appears that the substitution of one of the hydrogen atoms of the benzene or pyridine nucleus by a reactive group, such as —OH or —COOH, leads to a certain degree of susceptibility to microbial attack.

Pseudomonads and other Gram-negative rods have been shown to be capable of utilizing cyclic compounds as their sole source of carbon and energy by den Dooren de Jong (1926), Gray & Thornton (1928), Koser & Baird (1944) and Stanier (1948). It is interesting to note that although large numbers of motile Gram-negative rods were present in pyridine enrichment cultures, these organisms would not grow in pure culture in the pyridine media. Apparently *Proactinomyces* breaks down the pyridine molecule in such a way that the

products can satisfy the carbon, nitrogen and energy requirements of the Gram-negative rods as well as its own needs. Other organisms, reported by den Dooren de Jong (1926), Robbins (1917) and Ostroff & Henry (1939) to be capable of utilizing pyridine as a source of nitrogen and thus of rupturing the ring, could not satisfy their carbon and energy requirements with the remainder of the molecule, but needed an additional carbon compound, such as glucose. It appears from the evidence presented that, in pure culture, the utilization of pyridine as a sole source of carbon, nitrogen and energy is limited to organisms of the genus *Proactinomyces*, although certain other bacteria are able to grow in mixed culture with *Proactinomyces*, or to utilize pyridine as a source of nitrogen.

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## *Clostridium welchii* Iota Toxin: Its Activation by Trypsin

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**SUMMARY:** The isolation, from sheep, of strains of the Bosworth type of *Clostridium welchii* is recorded; it is proposed to designate this type as *Cl. welchii* type E.

The toxicity of culture filtrates of *Cl. welchii* type E after short growth periods is increased by treatment with crude trypsin preparations; no such increase can be demonstrated when the filtrates are from growths older than 5-7 hr. The toxin which is activated in these filtrates is the toxin iota. It appears that *Cl. welchii* type E strains elaborate prototoxin convertible by enzymes into iota toxin proper.

In 1948 Bosworth described a type of *Clostridium welchii* differing in toxigenicity from the four types A, B, C and D of Wilsdon's (1981) classification. It had been isolated from the intestine of a calf being examined for evidence of enterotoxaemia, and was characterized by the elaboration of a toxin which he designated iota (*i*). Subsequently we examined material from many cases of suspected enterotoxaemia in cattle and sheep, but it was not until 1944 that we isolated an iota-producing strain from the intestine of a lamb. Later in the same year iota toxin was identified in the bowel contents of three lambs, but, in spite of repeated attempts, no strains were isolated. In the spring of 1947, while examining a series of one hundred swab cultures taken from the udders of ewes on a farm infected with lamb dysentery, we identified iota toxin in forty of the mixed cultures from the swabs and isolated iota-producing strains from seven. These strains were tested by qualitative methods for the production of specific toxins and each was found to elaborate *Cl. welchii*  $\alpha$  and  $\theta$  toxins in addition to iota toxin. Each resembled *Cl. welchii* in morphology, cultural characters and staining reactions. They produced acid and gas in glucose, lactose, maltose, sucrose and glycerol, but only gas in inulin, mannitol and salicin. Bosworth included salicin among the sugars fermented by his strain; but this strain in our hands, when tested concurrently with the other strains, did not produce acid in this sugar. All the strains gave the typical *Cl. welchii* stormy fermentation reaction in litmus milk, and liquefied gelatine. When grown on the surface of Dorset egg medium and on coagulated serum, each strain produced a slight softening and pitting of the surface. The indole reaction was negative. As the strains thus resemble Bosworth's in all respects, we consider that they belong to his new type which, with his consent, we propose to designate type E.

In the course of testing these strains it was found by one of us (J.M.B.) that occasional filtrates of 5 hr. growths in a meat broth medium were non-toxic for mice when injected intravenously. When, however, the filtrates were treated with trypsin before injection they became toxic and, by antitoxin neutralization tests, were found to contain iota toxin. Other filtrates of similar growths contained only  $\alpha$  toxin before trypsin treatment; after treatment the  $\alpha$  toxin had disappeared and iota toxin was identified in the filtrates.

This suggested that iota toxin, like *Cl. welchii*  $\epsilon$  toxin, might be produced initially as a non-toxic precursor or prototoxin (Turner & Rodwell, 1948) convertible by enzyme action into a toxic form. Bosworth investigated the possibility that some such conversion might occur but found no increase in the toxicity of his filtrates after exposure to trypsin. These, however, in contrast to ours, were prepared from 18 hr. growths, and it seemed to us that at this time conversion from the non-toxic to the toxic form might have been completed. We therefore devised the following experiments to study the effect of trypsin on iota toxin.

#### MATERIAL AND METHODS

Filtrates were prepared from cultures of *Cl. welchii* type E after different periods of growth and examined for the amount of iota toxin before and after treatment with trypsin.

##### *The preparation of filtrates*

**Strains.** Four of the nine available strains were selected: CN 1241, Bosworth's strains; CN 1493, isolated from the intestine of a 6 months old lamb; CN 1870 and CN 1993, both isolated from swab cultures taken from the surface of ewes' udders.

**Medium.** Horse muscle infusion broth plus 1% by volume of papain digest of horse muscle (T.N. 0.3%), plus 30% (v/v) cooked meat particles. Volumes of 4 l. were used, steamed for 1 hr. at 100°, cooled to 37° and inoculated with 50 ml. of an overnight growth of seed culture in the same medium. Incubation was at 37°.

**Sampling.** Samples (100 ml.) were withdrawn by pipette during growth at 2, 8, 4, 5 and 7 hr. after inoculation. At 2 hr. growth, though slight, was always evident; the peak of growth, as judged by turbidity, a lightening in colour and gas evolution, was usually reached at about 5 hr.

**Filtration.** Samples were filtered immediately after collection through sterilizing filter-pads (Ford S.B. grade Sterimats).

**Treatment with trypsin.** A crude preparation of pig pancreas was used. It was kept at c. 4° at pH 4.0 and filtered through paper as required. The filtrate from the crude pancreas preparation was added (5% v/v) to culture filtrates without adjustment of the pH, and the mixtures incubated for one hour at 37°. The necessary dilutions were then made in saline and injected.

##### *Measurement of toxicity*

The toxicity of culture filtrates was measured by estimating the LD 50 for mice, and the minimal necrotizing dose (M.N.D.) for guinea-pigs. The LD 50 is defined as the smallest dose which, when injected intravenously into mice of 20–22 g. weight, kills 50% of the animals within 72 hr. The M.N.D. is the smallest dose producing a characteristic necrotic reaction when injected intracutaneously into guinea-pigs. Culture filtrates were diluted in saline and the volume of each dose under test was adjusted with saline to 0.5 ml. for the LD 50 and 0.2 ml. for the M.N.D. test.



As *Cl. welchii*  $\alpha$  toxin was almost certain to be present in filtrates from such short growths, and as by its lethal and necrotizing action it would interfere with the *in vivo* tests for iota toxin, an excess of  $\alpha$  antitoxin free from iota antitoxin was added to all dilutions.

In estimating both the LD 50 and the M.N.D. the dose in initial tests varied by 50 or 20 %. In confirmatory tests on the LD 50 they varied by 10 %; two or three LD 50 determinations were carried out on this range using two mice to each dose. The M.N.D. determination could not be made on a range closer than 20 %.

*The L + dose in mice.* The L + in mice is defined as the smallest dose of toxin which, when mixed with one unit of antitoxin, kills within 72 hr. 50 % of the animals injected intravenously. The unit of antitoxin was that devised by Mr A. T. Glenn in these Laboratories in 1948, and is contained in 0.004 ml. of a provisional standard serum prepared in a horse by the injection of filtrates of *Cl. welchii* type E. Serial dilutions of culture filtrate in 1.0 ml. were mixed with one unit of iota antitoxin in 1.0 ml. saline, and kept at room temperature for 80 min.; 0.5 ml. of each mixture was then injected into each of two mice. As in the LD 50 tests the dilutions varied initially by 20–50 %, and in confirmatory tests by 10 %. The L + dose was also determined with an excess of epsilon antitoxin (80 units) included in each iota toxin-antitoxin mixture.

## RESULTS

Iota toxin was not demonstrable in any of the four untreated culture filtrates from 2 hr. growths; after treatment with trypsin it was demonstrable in two (Table 1).

Table 1. *The effect of trypsin on the LD 50 (mouse) of iota toxin in culture filtrates after different periods of growth*

– T and + T = filtrate before and after treatment with trypsin.

| Period of growth (hr.) | Culture filtrates from |        |         |        |         |       |         |       |
|------------------------|------------------------|--------|---------|--------|---------|-------|---------|-------|
|                        | CN 1241                |        | CN 1870 |        | CN 1498 |       | CN 1998 |       |
|                        | – T                    | + T    | – T     | + T    | – T     | + T   | – T     | + T   |
|                        | LD 50 doses (ml.)      |        |         |        |         |       |         |       |
| 2                      | > 0.50                 | > 0.50 | > 0.50  | > 0.50 | > 0.50  | 0.09  | > 0.50  | 0.48  |
| 3                      | > 0.50                 | 0.09   | > 0.50  | 0.15   | 0.065   | 0.024 | 0.16    | 0.06  |
| 4                      | 0.21                   | 0.086  | 0.80    | 0.10   | —       | —     | —       | —     |
| 5                      | 0.12                   | 0.083  | 0.20    | 0.06   | 0.086   | 0.018 | 0.085   | 0.027 |
| 7                      | 0.05                   | 0.045  | 0.15    | 0.08   | 0.088   | 0.027 | 0.026   | 0.024 |

The LD 50 of two treated filtrates of 4 hr. growths, and of four filtrates of 5 hr. growths were less than those of the same filtrates before treatment with trypsin; the LD 50 of three out of four filtrates of 7 hr. growths did not show any significant change after treatment with trypsin.

Similar decreases in the M.N.D. after trypsin treatment of filtrates were

shown in the guinea-pig intracutaneous test (Table 2). The maximum dose (0.2 ml.) of the untreated filtrates of 2 hr. growths produced no reaction, but after treatment, 0.011 ml. of one filtrate and 0.04 ml. of another produced reactions characteristic of *iota* toxin. The M.N.D. of 3 hr. filtrates decreased to roughly one-third of their previous values after treatment, but the doses of 5 and 7 hr. filtrates were not significantly affected. Characteristic reactions did not develop as a rule until 48 hr. after injection. At this time the reactions consisted of flat, roughly circular areas of greyish white necrosis vaguely outlined with purple; occasionally the reactions consisted of irregular shaped areas of purplish haemorrhage without necrosis.

Table 2. *The effect of trypsin on the minimal necrotizing dose (M.N.D.) of iota toxin in culture filtrates after various periods of growth, measured in guinea-pig skin*

—T and +T = filtrate before and after treatment with trypsin.

| Period of growth<br>(hr.) | Culture filtrates from |        |         |        |
|---------------------------|------------------------|--------|---------|--------|
|                           | CN 1493                |        | CN 1993 |        |
|                           | —T                     | +T     | —T      | +T     |
|                           | M.N.D. (ml.)           |        |         |        |
| 2                         | > 0.20                 | 0.011  | > 0.20  | 0.04   |
| 3                         | 0.0065                 | 0.0024 | 0.006   | 0.0018 |
| 5                         | 0.0022                 | 0.0018 | 0.0018  | 0.0018 |
| 7                         | 0.0022                 | 0.0017 | 0.0014  | 0.0015 |

The L+ dose was also affected by treatment of the filtrates with trypsin although, like the M.N.D., only with filtrates from 2 and 3 hr. growths. The L+ for the 2 hr. untreated filtrates could not be determined because toxin could not be demonstrated in the maximum amount of filtrate that it was practicable to inject. After treatment with trypsin these filtrates had an L+ value of 1.2 ml. (Table 3).

Table 3. *The effect of trypsin on the L+ dose (mouse) of iota toxin in culture filtrates after various periods of growth*

—T and +T = filtrate before and after treatment with trypsin.  
NM = L+ not measurable because filtrate insufficiently toxic.

| Period of growth<br>(hr.) | Culture filtrates from                   |      |         |      |
|---------------------------|--|------|---------|------|
|                           | CN 1493                                  |      | CN 1993 |      |
|                           | —T                                       | +T   | —T      | +T   |
|                           | Volume (ml.) $\equiv$ 1 <i>iota</i> unit |      |         |      |
| 2                         | NM                                       | 1.2  | NM      | NM   |
| 3                         | 0.50                                     | 0.33 | 1.24    | 0.53 |
| 5                         | 0.33                                     | 0.33 | 0.36    | 0.33 |
| 7                         | 0.34                                     | 0.30 | 0.39    | 0.40 |

## DISCUSSION

Our results show that the toxicity of *Cl. welchii* type E filtrates is increased by treatment with crude trypsin preparations. As there is evidence that  $\alpha$  and  $\theta$  toxins are destroyed by this treatment, and the treated filtrates were neutralized by iota antisera not containing any  $\epsilon$  antibody, and not by  $\epsilon$  antisera containing no iota antibody, we conclude that the increased toxicity is due to iota toxin.

The increases of toxicity were not so great as those shown by  $\epsilon$  toxin (Batty & Glenny, 1947) but were sufficient to show that iota toxin, like  $\epsilon$  toxin, has a non-toxic precursor from which it is formed by the action of crude trypsin. We therefore postulate that type E strains produce, in addition to  $\alpha$  and  $\theta$  toxins, an iota prototoxin convertible by enzyme action into iota toxin proper. Judging by the results with  $\epsilon$  toxin reported by Batty & Glenny, the changes in the iota L+ value after trypsin treatment were far greater than would have been expected from the relatively small changes in the LD 50 dose.

The time at which the maximum increase in toxicity occurred could not be determined because the culture filtrates after the shortest growth periods tested were non-toxic, but it evidently must have been within the first 8 hr. after inoculation. It may be assumed that complete transformation from the suggested precursor had been brought about by the organism's own enzyme systems after 7 hr., as trypsin treatment then had no effect.

We wish to thank Prof. T. J. Bosworth for sending us his strain, and Dr C. L. Oakley for the supply of antitoxin and for much helpful advice and criticism.

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## The Phenomenon of Lysogenicity in Staphylococci

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**SUMMARY:** Of thirty coagulase-positive staphylococci, twenty-seven proved to be lysogenic. Free phage was found in filtrates of 4-5 hr. broth cultures of the lysogenic strains. The phages from six of these strains were examined by estimation of the amounts occurring in filtrates, for serology and for their range of lytic reactions with indicator strains. A number of strains were found which carried as many as five distinct bacteriophages. Probable phage mutations were also demonstrated. Interference effects due to lysogenicity were not a marked feature of the strains investigated.

It is assumed that the free phage released by the lysogenic strains is responsible for infection of sensitive indicator strains. The approximate proportion of lysogenic cells capable of releasing phage has been determined for three strains; it varied for the three strains and according to the indicator strains used, ranging from 1/3 cells inoculated to 1/30,000 cells inoculated. The mechanism of the release is probably due to multiplication of the phage in a number of lysogenic cells with consequent lysis of the cells and release of the phage.

Lysogenicity appeared to be a permanent feature of these strains. Every cell was apparently carrying phage and treatment with heat, with specific antibacteriophage serum, or by growth in broth containing sodium citrate did not make the cells non-lysogenic. For such strains, apart from an extracellular release of phage from a few cells in each culture, there is postulated an intracellular transference of phage to each daughter cell at cell division.

It has already been shown by Fisk (1942), Wilson & Atkinson (1945) and Williams Smith (1948*a, b*), that a large number of strains of coagulase-positive staphylococci are lysogenic; that is to say, when cultures of these strains are grown on agar plates together with strains sensitive to the bacteriophages present in the lysogenic cultures, evidence of lytic action appears and the bacteriophages responsible may be isolated.

Williams Smith examined twenty-two strains used by Wilson & Atkinson as propagating strains for their typing phages, and found seventeen of them to be lysogenic. By means of cross-resistance tests carried out according to Bail's technique, he showed that the range of phages lysing a given strain could be restricted by the development of resistant variants of the strain. These variants were often lysogenic; this was clearly shown in the case of non-lysogenic cultures which developed lysogenic, resistant variants. He concluded that the carriage of a phage by a given strain precluded its lysis by that phage. He was able to alter the pattern of phage reactions of certain strains by growing two different lysogenic cultures together in broth for prolonged periods: the altered strains presumably acquired the phage of the strain with which they had been growing. He was of the opinion that acquired phage resistance was responsible for the classification of many originally identical strains as different phage types.

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Since the observation (Rountree, 1947*a*) of two mutually lysogenic cultures which were apparently carrying identical bacteriophages did not agree with the interference effects reported by Williams Smith, further investigations have been made on the phenomena of lysogenicity in the staphylococci. There are also contradictory reports in the literature on the presence of free phage in filtrates of broth cultures of staphylococci. Callow (1922) stated that she could obtain bacteriophage by the filtration of staphylococcal cultures. Burnet & Lush (1936) described a lysogenic mutant of a white staphylococcus which liberated bacteriophage in young and old cultures. On the other hand, Fisk (1942) and Williams Smith (1948*b*) failed to demonstrate the presence of bacteriophage in filtrates of lysogenic cultures.

The phenomena of lysogenicity as exhibited by the staphylococci may throw some light on the problems associated with the modes of reproduction of latent viruses generally, whether in bacterial or animal cells. An attempt has therefore been made to demonstrate the mechanisms determining the maintenance of the lysogenic state in the staphylococcal cell and the infection of indicator strains when placed in contact with lysogenic cultures.

#### MATERIALS

Thirty-four strains of coagulase-positive staphylococci, producing various amounts of pigment and of  $\alpha$  and/or  $\beta$  toxin, were used.

(a) Twenty-one strains used in this laboratory for the propagation of the typing bacteriophages.

(b) Two strains used in the serological differentiation of staphylococci, Christie & Keogh's type 5 culture (1940) and 789, a culture identical with Cowan's type II (1939).

(c) Five lysogenic strains, S14, S17, S21, S33 and S35 (Rountree, 1947*a*).

(d) Two lysogenic strains, S58, and S59, isolated from human infections.

(e) Four strains, S11, S50, B83 and R192, whose lysogenicity was not investigated but which served as indicator strains sensitive to certain of the bacteriophages encountered.

#### RESULTS

##### *Presence of bacteriophage in filtrates of lysogenic cultures*

A number of previous experiments had confirmed the fact that bacteriophage could not be demonstrated in filtrates of 18 hr. broth cultures of known lysogenic strains. However, it was found that phage could be demonstrated in broth cultures of such strains by the following method. A fairly heavy inoculum of cells was grown in Todd-Hewitt (1932) broth at 37° for 4–5 hr., after which period obvious turbidity had developed. The cultures were centrifuged for 10 min. at 8000 r.p.m. and filtered through gradocol membranes of an A.P.D. of 0.84  $\mu$ . The filtrates were then tested for bacteriophage against a series of strains known to be sensitive on agar plates to the lysogenic strain. Free phage was detected in filtrates from twenty-five of the thirty strains tested (Table 1). In the three strains 18, 284 and 1851, in which no free phage

could be detected, it is possible that the available indicator strains were not suitable. In two other strains, S14 and S21, known to be lysogenic on agar, no free phage could be detected. Filtrates of S14 were also tested to see if they contained any inactivating agent for the phage (14/17) carried by this strain, but no evidence of inactivation could be found when such filtrates were mixed with phage 14/17 and incubated at 37° for 2 hr.

Table 1. *Indicator strains of Staph. aureus sensitive to bacteriophages carried by twenty-seven lysogenic strains of Staph. aureus*

| Lysogenic strain | Indicator strains        | Lysogenic strain | Indicator strains        |
|------------------|--------------------------|------------------|--------------------------|
| 3                | 36, S59                  | 1363             | 18, R192                 |
| 4                | S50, S59                 | 1670             | 36, R1760, R192          |
| 33               | S59, B83                 | R1760            | 3, 36, S59               |
| 36               | 3, 1307, S59             | 2329             | 3, S59                   |
| 144              | 18, 373, S59, B83        | S14              | S17                      |
| 145              | 211, 284, 1339, S17, CK5 | S17              | 211, 284, 1339           |
| 211              | 284, 1339, CK5           | S21              | S11                      |
| 873              | 2329                     | S33              | 3, S35                   |
| 761              | 3, 18                    | S35              | 3, S33                   |
| 925              | 2329                     | S58              | S50, S59, B83            |
| 987              | 3, 36, 1351, S59         | S59              | 3, R1760, 2329           |
| 1163             | 3, 36, S59               | CK5              | 145, 211, 284, 1339, S17 |
| 1307             | 3, R1760                 | 789              | 145, 211, 284, 1339      |
| 1339             | 145, 211, S17, CK5, 789  |                  |                          |

Exact estimations of the amount of phage/ml. of each filtrate were not made in all cases, but in those in which it was carried out there was a wide variation from strain to strain, the counts varying from 50 to 7,000,000 phage particles/ml.

There were many cases of mutual lysogenicity among the thirty strains; for example, 3 and S59, 3 and 36, R1760 and S59, S33 and S35, 145 and 1339, and 211 and 1339. In all cases, the filtrates from lysogenic strains produced no plaques when tested on agar plates inoculated with the strains used for the preparation of the filtrates.

Burnet & Lush (1935) showed that the staphylococcal bacteriophages which they classified as strong or intermediate were absorbed by all 'aureus' staphylococci tested, whether they lysed these strains or not. This was confirmed for the phages used in the typing of staphylococci (Rountree, 1947*b*). The failure to demonstrate free bacteriophage in older cultures of lysogenic strains may, therefore, be ascribed to its absorption on to the bacterial cells. In the two lysogenic strains from which no detectable phage was released in broth cultures, it is possible that the phage was more quickly reabsorbed than was the case with the other strains, since it was later shown that S14 probably did release free phage.

#### *Bacteriophage carried by six closely related strains*

From the thirty strains, seven were selected which were closely related, both in their reactions with the typing phages and in their lysogenic interreactions. These were 145, 211, 284, 1339, S17, CK5 and 789. Strain 284 was not detectably lysogenic; the other six strains were (Table 2).

The bacteriophages carried by each of these six strains were prepared against the susceptible strains listed in column 8 of Table 2, making a total of twenty-five phages. They were isolated from plates made by Fisk's method, re-isolated at least twice from single plaques and the filtrates prepared in the usual way. The dilution of bacteriophage filtrate just giving confluent lysis on agar with its homologous staphylococcus was tested against all seven strains. The serological type of the bacteriophages was also determined, since it has been shown (Rountree, 1949) that two serological groups (A and B) can be

Table 2. *The reactions with typing phages and the lysogenic interreactions of seven closely related strains of Staph. aureus*

| Strain no. | Reactions with typing phages | Strains susceptible to phage carried by the strain |
|------------|------------------------------|--|
| 145        | 3B +, 51 + + + +             | 211, 284, 1339, S17, CK 5                          |
| 211        | 3B + + + +, 3C + +           | 284, 1339, CK 5                                    |
| 284        | 3A + + + +, 3B +, 3C +       | —  |
| 1339       | 3B +, 3C + + + +             | 145, 211, S17, CK 5, 789                           |
| S17        | 3B +, 3C +, 14 + + + +       | 211, 284, 1339                                     |
| CK 5       | 3B + +, 3C + +               | 145, 211, 284, 1339, S17                           |
| 789        | 3B + + + +                   | 145, 211, 284, 1339                                |

+ + + + = confluent lysis; + + = uncountable discrete plaques; + = 10-20 plaques.

distinguished among the typing phages and that a third group (F) is also found among these lysogenic cultures. In addition, one phage (S17/211) was found which was not neutralized by any of the available sera. In Table 3 are set out the lytic and serological reactions of each of the twenty-five phage preparations. The bacteriophage preparations are designated by the number of the culture of their origin followed by the number of the strain on which they have been propagated; e.g. 145/211 indicates phage from strain 145 grown on strain 211. They are arranged in the order of the lysogenic strains from which they originated. It is clear that some of the bacteriophages are identical and that a total of twelve distinct phages were obtained from the six strains. For example, the phages derived from strains 145, 211, S17, CK 5 and 789, which lyse 284, are identical; phages 145/211, 1339/211, 1339/CK 5 and 1339/789 are identical; phage 145/CK 5 and 211/CK 5 are identical and so are phages 145/S17 and 1339/S17.

Filtrates of the lysogenic strains grown in broth were examined by plating each on all seven indicator strains, and the phage content/ml. of each filtrate was determined. Each separate phage was isolated from these filtrates and tested in the same manner as the series from the Fisk plates. Individual phages from the two sets of preparations in all cases gave corresponding reactions. Table 4 gives the number of plaques/ml. of the various filtered broth cultures which developed when the filtrates were titrated with the seven strains.

Other interesting points are revealed in Tables 3 and 4. The lysogenic strains are each carrying more than one bacteriophage. From strain 145 five phages were obtained, varying in their lytic reactions, in serological type, and in the amount of each present in a filtrate. Strain 211 produced three phages

of the same serological group differing in lytic patterns and in their titres on sensitive strains. Strain 1339 carried three phages, the group F phage lysing strain 145 being different from that lysing strains 211, CK 5 and 789. It should

Table 3. *The serological and lytic reactions of twenty-five bacteriophages isolated from six lysogenic strains of staphylococci*

| Bacteriophage | Serological type | Lytic reactions with indicator strains |     |     |      |     |      |     |
|---------------|------------------|--|-----|-----|------|-----|------|-----|
|               |                  | 145                                    | 211 | 284 | 1339 | S17 | CK 5 | 789 |
| 145/211       | F                | CL                                     | CL  | tr. | +    | CL  | CL   | CL  |
| 145/284       | B                | —                                      | —   | CL  | —    | tr. | —    | —   |
| 145/1339      | B                | tr.                                    | +   | +   | CL   | +   | CL   | +   |
| 145/S17       | A                | —                                      | tr. | —   | CL   | CL  | —    | —   |
| 145/CK 5      | B                | ++                                     | +   | ++  | SCL  | +   | CL   | —   |
| 211/284       | B                | —                                      | —   | CL  | —    | tr. | —    | —   |
| 211/1339      | B                | +                                      | +   | +   | CL   | +   | CL   | +   |
| 211/CK 5      | B                | ++                                     | +   | ++  | SCL  | +   | CL   | —   |
| 1339/145      | F                | CL                                     | tr. | tr. | —    | tr. | CL   | tr. |
| 1339/211      | F                | CL                                     | CL  | —   | +    | CL  | CL   | CL  |
| 1339/S17      | A                | —                                      | +   | —   | CL   | CL  | —    | —   |
| 1339/CK 5     | F                | CL                                     | CL  | tr. | CL   | CL  | CL   | CL  |
| 1339/789      | F                | CL                                     | CL  | tr. | CL   | CL  | CL   | CL  |
| S17/211       | Not A, B or F    | —                                      | CL  | tr. | tr.  | tr. | +    | —   |
| S17/284       | B                | tr.                                    | tr. | CL  | tr.  | tr. | tr.  | tr. |
| S17/1339      | B                | tr.                                    | tr. | tr. | CL   | tr. | tr.  | tr. |
| CK 5/145      | F                | CL                                     | tr. | tr. | +    | tr. | SCL  | —   |
| CK 5/211      | B                | CL                                     | CL  | +   | CL   | —   | +    | —   |
| CK 5/284      | B                | —                                      | —   | CL  | —    | —   | —    | —   |
| CK 5/1339     | B                | CL                                     | CL  | tr. | CL   | —   | tr.  | —   |
| CK 5/S17      | A                | tr.                                    | tr. | tr. | ++   | CL  | —    | —   |
| 789/145       | B                | CL                                     | +   | SCL | CL   | tr. | SCL  | —   |
| 789/211       | B                | CL                                     | CL  | +   | CL   | —   | +    | —   |
| 789/284       | B                | —                                      | —   | CL  | —    | —   | —    | —   |
| 789/1339      | B                | ++                                     | CL  | +   | CL   | +   | +    | +   |

++ = numerous discrete plaques.

CL = confluent lysis.

+ = 10–20 plaques.

SCL = semi-confluent lysis.

tr. = < 10 plaques.

Table 4. *Number of plaques/ml. of filtrate of lysogenic strains developing on various indicator strains*

| Filtrate of lysogenic strains | No. of plaques/ml. of filtrate on indicator strains |         |        |           |        |       |     |
|-------------------------------|---|---------|--------|-----------|--------|-------|-----|
|                               | 145   | 211     | 284    | 1339      | S17    | CK 5  | 789 |
| 145                           | 0   | 200     | 8,500  | 80,000    | 250    | 1,400 | 0   |
| 211                           | 0   | 0       | 500    | 500,000   | 0      | 1,400 | 0   |
| 1339                          | 85,000  | 10,000  | 0      | 0         | 10,000 | 3,000 | 500 |
| S17                           | 0   | 20,000  | 2,500  | 7,000,000 | 0      | 0     | 0   |
| CK 5                          | 5,800,000   | 176,000 | 10,000 | 750,000   | 1,250  | 0     | 0   |
| 789                           | 95,000  | 143,000 | 200    | 250,000   | 0      | 0     | 0   |

be noted that the phages 1339/211, 1339/CK 5 and 1339/789 will also lyse S17, and that 1339 is carrying two phages, both of which can be isolated on S17. The 1339/S17 phage of group A is more specific than the group



F phage. From strain S17 three phages were obtained in different amounts and of two serological groups. Similarly, strain CK5 gave phages of four lytic patterns and three serological groups. Phages CK5/211 and CK5/1839 may be identical, the difference in titre when the filtrate was titrated on 211 and 1839 being possibly due to a certain proportion of resistant cells in strain 211. From strain 789, phages of one serological group and four different lytic patterns were obtained.

It has already been shown (Rountree, 1949) that strain 86 carries bacteriophages belonging to two serological groups, so it is evident that the phenomenon of multiple lysogenicity is not confined to this particular group of strains; it is probably widespread among the staphylococci.

It may be questioned whether these phages have been modified by their passage through the indicator strains, since they are of necessity examined after their propagation in strains sensitive to them. However, some strains in the series are lysed by a number of clearly different phages; for example, strain 211 is lysed by the typing phage 3B which belongs to serological group A, and by three other phages of different lytic pattern, namely, those from strains 145 and 1839 (group F), from strain S17 (not group A, B or F) and from strains CK5 and 789 (group B). This suggests that, even if modification during passage does occur, its direction is governed not by some characteristic of the staphylococcus such as lysogenicity, but by the intrinsic character of the phage itself.

Furthermore, it can be shown that when two serological types of bacteriophage are isolated from a given strain, filtrates of such strains before passage of the phages in the sensitive strains do in fact contain the two serological types of phage. For example, the filtrate of S17, which contains group B phage lysing 1839 and 284 and a phage not of group A, B or F that lyses 211, loses only its group B phage activity on the addition of an appropriate amount of group B phage antiserum. After incubation at 37° for 4 hr., the mixture on plating contains the original amount of phage active against 211, although phage lysing 1839 or 284 can no longer be demonstrated.

It will be noted that with many of the phages trace reactions, that is, the development of a very few plaques on certain strains, occurred frequently. Since the phages, with the exception of those lysing strain 284, had been grown on lysogenic cultures, it is likely that in some cases these trace reactions were due to contamination of the phage preparation with phage derived from the propagating strain and were not necessarily due to mutation of the phage. An example of this is found in the phage from strain 1839, which on isolation in filtrates has no action on 284. This phage, after growth in strains 145, CK5 and 789 which carry phage lysing 284, gave a few plaques with 284; such plaques may be due to phage released from 145, CK5 and 789 during lysis.

In certain cases, however, it is possible that mutation took place. All the phages propagated on 284 gave identical serological and lytic reactions, being specific for this strain and belonging to serological group B. For the five filtrates in which this phage was found, the ratio of the total amount of group B phage present in the filtrate to the amount of group B phage lysing

284 was calculated from the values given in Table 4 (Table 5). In the filtrates of strains 211, S17 and 789 containing only small amounts of the phage lysing 284, the ratios (1000 to 1, 8000 to 1 and 2400 to 1 respectively) suggest the possibility of the mutation of the group B phage. Whether this mutation takes place during the release of the phage from the lysogenic cells or during its passage with 284 cannot be determined.

Table 5. *The ratio in various filtrates of the total amounts of type B phage to the amount of type B phage specific for strain 284*

| Filtrate of strain | Type B phage/ml. (a) | Phage lysing<br>284/ml. (b) | Ratio a/b |
|--------------------|----------------------|-----------------------------|-----------|
| 145                | 90,000               | 8,500                       | 10.6      |
| 211                | 500,000              | 500                         | 1,000     |
| S17                | 7,000,000            | 2,500                       | 3,000     |
| CK 5               | 940,000              | 10,000                      | 94        |
| 789                | 480,000              | 200                         | 2,400     |

Lysogenic strains are sensitive to phages very closely related to those which they carry. For example, the phage 145/211, the phages 1839/S17, 1839/CK 5 and 1839/789, and the phage CK 5/145, all appear, after passage of the phage through a sensitive strain, to be modified in infectivity, so that the previously resistant cells of the parent become sensitive to them. The alteration in infectivity is not accompanied by any other detectable changes. It seems that the results previously reported (1947*a*) with cultures S33 and S35 and their phages were due to this alteration in infectivity by passage. Phage from S33 or S85 when isolated by filtration of the culture produces plaques on the sensitive strain, but not on the strain from which it has been prepared. However, after passage of the phage from S33 through S35, and vice versa, both phages will lyse both S33 and S35 to titre, and no lytic or serological difference can be detected between them.

#### *Infection of sensitive strains by bacteriophage from lysogenic cultures*

By filtration of young broth cultures it has been shown that lysogenic staphylococci release free phage into the surrounding medium. It can therefore be assumed that infection of sensitive strains placed in contact with lysogenic strains is due to the action of this free phage. Questions arise, however, as to what proportion of cells in a lysogenic strain are able to initiate such infection and what mechanism is operative in the release of the phage from the lysogenic cell.

The first question may be answered by the following observations. When 0.02 ml. of a suitably diluted young broth culture of a lysogenic strain is spread on agar plates and then covered with an inoculum of a sensitive indicator strain sufficient to give confluent growth, the number of plaques developing in the sensitive strain may be counted after incubation. At the time of inoculation of the plates, a viable count of the number of lysogenic cells is also made. It is then possible to calculate the ratio of lysogenic cells inoculated to the number of plaques developing. It should be noted that this ratio does

not necessarily indicate the number of cells of the lysogenic culture which are capable of infecting the indicator strain, since under the conditions of the experiment the number of cell divisions which may take place in the lysogenic cells before infection occurs cannot be ascertained. The method is also not very precise because multicellular aggregates of staphylococci giving rise to one colony in the viable count are assumed to have arisen from one coccus. However, the approximate values obtained indicate the order of magnitude of the number of cells initiating infection of a susceptible strain, and therefore presumably capable of releasing bacteriophage. These values have been

Table 6. *The number of viable cells of lysogenic strains capable of producing one plaque when tested with certain indicator strains*

|                  |        | Lysogenic strain                          |   |                     |
|------------------|--------|---|---|---------------------|
| Indicator strain | No.    | Inoculum                                  | Plaques   | Ratio<br><i>a/b</i> |
|                  |        | 10 <sup>6</sup> cells/ml.<br>( <i>a</i> ) | developing<br>10 <sup>6</sup> /ml. ( <i>b</i> ) |                     |
| 1339             | S17    | 4,800                                     | 1,800   | 2.7                 |
| 211              | S17    | 4,800                                     | 17  | 280                 |
| 284              | S17    | 4,800                                     | 0.16  | 30,000              |
| 145              | 1339   | 40,000                                    | 45  | 900                 |
| 211              | 1339   | 40,000                                    | 110   | 360                 |
| S17              | 1339   | 40,000                                    | 1,400   | 28                  |
| S17              | S14/1  | 1,000                                     | 1   | 1,000               |
|                  | S14/2  | 750                                       | 4.5   | 170                 |
|                  | S14/3  | 5,200                                     | 13  | 380                 |
|                  | S14/4  | 6,000                                     | 15  | 400                 |
|                  | S14/5  | 6,000                                     | 12.5  | 480                 |
|                  | S14/6  | 2,650                                     | 9   | 290                 |
|                  | S14/7  | 2,000                                     | 6   | 340                 |
|                  | S14/8  | 850                                       | 2   | 420                 |
|                  | S14/9  | 3,250                                     | 2   | 1,600               |
|                  | S14/10 | 1,500                                     | 4.5   | 300                 |
|                  | S14/11 | 17,700                                    | 66  | 270                 |

determined for three strains: S17 against three indicator strains, 1339 against three indicator strains and S14 against S17. The ratio was also determined for eleven single-colony cultures of strain S14. In Table 6 the number of cells inoculated and of plaques developing have been expressed as numbers/ml. of the broth culture used; the final column gives the number of cells in the inoculum producing one plaque.

Three different values were obtained with strain S17 according to the indicator strains used, and these values correspond well with the relative amounts of the three phages present in the filtrates of this strain (Table 4). The value for phage lysing 1339 is exceptionally high. Three different values were also obtained for strain 1339. The values for the eleven cultures of S14 varied from one plaque/170 cells inoculated to 1/1600, and are probably within the limits of error of the method.

The conclusion may be drawn that phage is released only from a certain proportion of cells in a given lysogenic strain. This proportion is fairly constant for any given strain, but it varies from phage to phage when more than one

phage occurs in a particular strain. The figures do not, however, indicate whether a *single* cell of a strain that produces more than one type of phage may at a given moment itself produce more than one type. Further, it is not possible to say definitely whether the phage is released by lysis of a small percentage of cells or whether phage may be extruded from cells which remain viable. Strain S17, which produces large amounts of phage active against 1339, would be a convenient strain for an investigation of this problem by single-cell culture methods.

### *Permanence of lysogenicity*

In general terms it may be stated that bacteriophage may be detected in every cell of a lysogenic strain of staphylococcus, and that no treatment has so far been successful in making the strain non-lysogenic. The strains in this collection were grown in broth for periods varying from 1 to 5 years and during this time were plated and repicked from single colonies on numerous occasions; yet the twenty-seven lysogenic strains are still lysogenic. On one occasion, strain S17 was plated out, and a hundred of the resulting single colonies were picked into broth and retested for lysogenicity. All proved to be lysogenic. Numbers of single colonies from other strains in the series have also been tested with similar results.

Some of the bacteriophages carried by these strains were sensitive to heat when in cell-free filtrates, being destroyed by heating at 49° for 1 hr. When, however, the lysogenic strains carrying these phages were heated at this temperature for 2 hr. and replated, all the resultant colonies proved to be lysogenic.

Some of the bacteriophages carried by these strains are citrate-sensitive, being unable to lyse sensitive strains in the presence of 1% sodium citrate. When, however, the lysogenic strains carrying these citrate-sensitive phages were grown for 7–10 days in broth containing 1% sodium citrate and were plated at intervals during this incubation period, bacteriophage could be demonstrated in all single colonies tested.

Finally, growth of a lysogenic strain in serum containing specific antibodies for the bacteriophage carried by the strain did not remove the lysogenicity. Very small inocula of strains S14, S17 and 1339 suspended in broth with equal volumes of undiluted high-titre serum were each incubated for 4–5 hr. at 37° until turbidity could be detected and were plated out. Every colony tested after incubation overnight was lysogenic, up to eighty colonies from a single plate being tested on various occasions.

### DISCUSSION

The results clearly indicate that the association of bacteriophage and lysogenic cell is very intimate. Phage is to be found in each single bacterial colony from a lysogenic staphylococcal strain. Since agents such as heat and antiserum, which destroy the bacteriophage when it is in the free state, do not alter the lysogenicity of the strain, an intracellular method of passage of the phage in such strains is postulated such that the daughter cells of each staphylococcal

fission each receive at least one phage particle. It is possible that bacteriophage, which is predominantly nucleoprotein in composition, is associated with the nuclear apparatus of the lysogenic cell in such a way that regular distribution of the phage to the daughter cells occurs at cell division.

The relation between bacteriophage and lysogenic cell is, however, variable, since only a certain proportion of cells in each lysogenic strain liberates bacteriophage. This proportion varies from strain to strain and may be very small. The usual proportion appears to be of the order of 1 cell in every 1000. No examples have been found in which every cell in a strain releases phage. Two mechanisms of the release may be postulated. Either the phage is extruded by cells which remain viable; or the modal equilibrium between phage and cell is unstable, and in a certain proportion of the infected cells the phage multiplies as it does in sensitive cells; as a consequence the cell is lysed and its phage liberated. Since this second postulate has as its basis a known method of phage liberation, whereas the first is highly speculative, liberation by lysis is the most probable method of release of phage from lysogenic cells. The fact that liberated phage, after passage through a sensitive strain but apparently otherwise unaltered, can be shown in a number of cases to be fully infective for all the cells of the original lysogenic strain, may be significant in this connexion. Burnet & Lush (1936) found indications that the liberation of their mutant C' lysogenic phage from ageing cultures was always associated with the destruction of the coccus from which it was derived.

The data in Tables 1 and 3 make it clear that the possession of lysogenicity does not render the staphylococci resistant to the lytic action of bacteriophages. Of the twenty-seven strains listed in Table 1, only two have so far proved resistant to the action of the phages available. As pointed out by Williams Smith (1948*a*), the results obtained with the staphylococci cannot be explained on the basis of the 'penetration' hypothesis postulated by Delbrück (1945) for the *Bacterium coli* T bacteriophages. The material presented in this paper also suggests that interference effects are very limited among staphylococci, many of which show multiple lysogenicity, and may consist solely of inhibition of lysis by the bacteriophage carried by the strain, not extending even to very closely related or apparently identical phages. The possession of lysogenicity may be only partly responsible for the various reaction patterns obtained when a series of typing phages acts upon a number of closely related staphylococci.

Williams Smith (1948*a, b*) showed that after the development of resistance to various phages derived from lysogenic strains, originally non-lysogenic strains became lysogenic and were changed in their reactions to a number of typing phages. The reactions of such variants were similar to those of the lysogenic strains producing the resistance-inducing phages. He concluded that acquired phage resistance was responsible for the classification of many initially identical strains as different phage types. In certain sensitive strains and in some resistant strains, however, he was unable to obtain evidence of lysogenicity. My results suggest even greater complication. The strains differ in their reactions to the typing phages, but the typing phages belong to a serological group different from that of the majority of the phages carried by these

strains. The typing phages acting on these strains belong to serological group A (Rountree, 1949), while the sole phage of this serological character which has been isolated from only some of these strains lyses strain S17 and is restricted in its activity. The differing reactions given by the strains with the typing phages cannot, therefore, be attributed directly to the fact that they are carrying one or more of these typing phages. Furthermore, strain 284, which showed a very narrow phage specificity, was apparently not lysogenic.

The intrinsic characters of the staphylococcal cell which determine sensitivity to specific bacteriophages is still unexplained, but the available evidence suggests that it is not primarily due to the possession of lysogenicity.

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## The Serological Differentiation of Staphylococcal Bacteriophages

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**SUMMARY:** By the use of anti-bacteriophage sera prepared in rabbits, thirty-nine staphylococcal phages were divided into six serological groups. The first group (A) comprised phages lysing coagulase-positive staphylococci of human origin. They were stable at 20° but inactivated at 49°. They multiplied in broth cultures containing sufficient tryptophan but rarely produced clearing of such cultures. The second group (B) lysed both bovine and human coagulase-positive staphylococci. They were markedly sensitive to heat and required growth factors present in the vitamin B complex. Group C comprised phages of ovine origin which were antigenically related to group B phages and also resembled them in their growth requirements. Group D comprised phage K, which lysed both coagulase-positive and negative staphylococci and was antigenically related only to phage W. Phage W belonged to group E and lysed only some coagulase-negative staphylococci. Group F was related in its general characters with the phages of group A.

A staphylococcus was found carrying two serologically distinct phages, one of which was detected during the process of adaptation of a phage filtrate to a new propagating strain.

Since many strains of staphylococci are lysogenic, lytic filtrates may contain contaminating phages which manifest themselves during adaptation. Adequate serological characterization of the phages used for typing and for investigations of phage-bacterium relationships and of apparent mutation is therefore necessary.

Antisera to bacteriophages have been a valuable tool for the definition of the relationships of various races of phages (Burnet, 1933; Craigie, 1940). The only serological investigation of staphylococcal phages is that of Burnet & Lush (1935) who examined a series of thirteen staphylococcal phages and by serological and other methods distinguished six groups among them. Since the introduction by Wilson & Atkinson (1945) of their method of phage-typing staphylococci, a larger series of phages has become available for examination. This communication deals with the serological examination of these phages. In the series there has also been included a number of other staphylococcal phages whose reactions are of interest. The results indicate that the serological characteristics of the phages are associated with other properties such as stability and the range of organisms lysed.

### MATERIAL AND METHODS

*The bacteriophages.* The bacteriophages used were as follows.

(a) The series originally described by Wilson & Atkinson for the typing of staphylococci of human origin. Their nomenclature has since been simplified by omitting the designation of the propagating strain of staphylococcus, and,

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in cases where phages were developed by adaptation, by giving to the adapted phage the number of the original phage plus a suitable letter; e.g. 3/211 has become 8B and 3/1389 has become 8C. A number of adapted phages has been added to the original collection.

(b) Three phages, X2, G12 and S7, lysing staphylococci pathogenic to sheep and isolated by Dr Williams Smith.

(c) The phage K of Kreuger & Northrop (1930-1) is phage Au2 in Burnet & Lush's series. For an account of its history Burnet & Lush's paper should be consulted. It is probable that this is the polyvalent phage used by a number of workers.

(d) Phages 21 and W isolated in Australia (Rountree, 1947*a, b*).

(e) Eight phages obtained from cultures examined during an investigation of lysogenicity; phages 145/211, CK 5/145, S17/211 and phage from strain 1389 propagated on five different strains.

*Anti-bacteriophage sera.* Sera were prepared in rabbits against phages 29, 51, W, X2 and 1389. The usual course of injections of the phage filtrates was 0.5, 0.8, 1.0, 1.5 and 2.0 ml. at 5-day intervals, the rabbits being bled 7-9 days after the last injection. I am indebted to Dr Elford for a supply of anti-K serum and to Dr Williams Smith for a supply of anti-42D serum.

*Phage neutralization tests.* These were carried out by the method of Burnet (1933). Dilutions of the sera, usually tenfold, were made in nutrient broth and an equal volume of diluted phage added to each tube. The phage dilution used was that which had been previously shown to give a countable number (50-200) of plaques when 0.02 ml. quantities were plated. The phage-serum mixtures were incubated at 37° for 4 hr. and 0.02 ml. quantities plated in duplicate on the appropriate propagating strain of staphylococcus. After incubation overnight at 37° the number of plaques was counted. The titre of the serum was expressed as the reciprocal of that dilution which gave approximately 80 % reduction in the plaque count as compared with that given by controls consisting of phage diluted with normal rabbit serum or broth.

## RESULTS

### *Phage neutralization tests*

Titrated with their homologous phages, the serum titres were: 20,000 for 29, 42D, 51 and 1389; 10,000 for X2; 100,000 for K; and 200,000 for W.

The neutralizing power of these sera was tested with thirty-nine staphylococcal phages (Table 1). Every phage was tested against serum 51, but when, during the course of the work, it became apparent that definite groups of phages could be distinguished which did not react with sera neutralizing other groups, only a few representative phages in each group were tested. The results show that it is possible to divide the phages into six serological groups, which for purposes of convenience have been designated A, B, C, D, E and F.

Group A contains the phages 3A, 8B, 3C, 6, 7, 14, 42B, 42E, 47, 47A, 47B, 47C, 51 and R1760. These are neutralized by serum 51 and not by the other sera. The serum titre is slightly lower (10,000) against phages 42E, 47, 47A



and 47 B, indicating either minor antigenic differences or some variation in the degree of reactivity of these four phages. As a whole, however, the phages may be considered to form a serologically homogeneous group.

Group B contains phages 29, 29 A, 31, 31 A, 44, 44 A, 52, 52 A, AH 2941, 42 C and 42 D. They are neutralized by sera 29 and 42 D, and although the neutralization titres vary, the group as a whole is serologically differentiated. Sera against 29 and 42 D were used as it was thought that they might reveal differences in these phages, since phage 29 was isolated from a staphylococcus

Table 1. *The neutralization of staphylococcal bacteriophages by antiphage sera*

| Group | Bacteriophages   | Anti-sera against |        |        |        |         |         |          |
|-------|--|-------------------|--------|--------|--------|---------|---------|----------|
|       |  | 51                | 29     | 42 D   | X 2    | K       | W       | 1839/789 |
| A     | 8 A, 3 B, 3 C, 14, 51, 6, 7,   | 20,000            | 0      | 0      | 0      | 0       | 0       | 0        |
|       | 42 B, 47 C, R 1760   |                   |        |        |        |         |         |          |
|       | 42 E, 47, 47 A, 47 B   | 10,000            | —      | —      | —      | —       | —       | —        |
| B     | 29   | < 20              | 20,000 | 1,000  | 200    | tr.     | 0       | —        |
|       | 29 A   | < 20              | 20,000 | 20,000 | —      | —       | —       | —        |
|       | 31   | 20                | 20,000 | 10,000 | —      | —       | —       | —        |
|       | 31 A   | < 20              | 10,000 | 20,000 | —      | —       | —       | —        |
|       | 44, 44 A   | 20                | 10,000 | 10,000 | —      | —       | —       | —        |
|       | 52   | < 20              | 20,000 | 10,000 | —      | —       | —       | —        |
|       | 52 A   | 20                | 20,000 | 2,000  | —      | —       | —       | —        |
|       | AH 2941  | tr.               | 1,000  | 20,000 | —      | tr.     | —       | —        |
|       | 42 C, 42 D   | < 20              | 4,000  | 20,000 | 200    | 0       | tr.     | 0        |
| C     | X 2  | 0                 | 2,000  | 200    | 10,000 | 0       | 0       | 0        |
|       | G 12, S 7  | 0                 | 1,000  | 200    | 1,000  | 0       | —       | —        |
| D     | K  | 0                 | 0      | 0      | 0      | 100,000 | 200     | 0        |
| E     | W  | 0                 | 0      | 0      | 0      | 20,000  | 200,000 | tr.      |
| F     | 1839/789, 1839/CK 5,<br>1839/145, 1839/S 17,<br>1839/211, 145/211,<br>CK 5/145, 21 | 0                 | 0      | tr.    | 0      | 0       | 0       | 20,000   |

The results are expressed as the reciprocals of the serum dilutions neutralizing 80 % of the phage; tr. indicates < 80 % neutralization by a 1/2 dilution of serum.

of human origin, whereas phage 42 D, although first propagated on a strain isolated from an outbreak of human staphylococcal food-poisoning, was found by Dr Williams Smith to lyse many strains from bovine mastitis. The results indicate that these two phages are not identical but are closely related. The phages in this group cross-reacted slightly with serum 51 and more markedly with serum X 2. This overlap with X 2 is of interest in view of the animal origin of some of the strains lysed by 42 D and the animal origin of phage X 2.

Group C consists of the three ovine phages X 2, G 12 and S 7. All three cross-react with sera 29 and 42 D and appear to be fairly closely related antigenically to the phages of group B. The differences, however, are great enough to justify their separation as a distinct serological group. There are differences also between X 2 and the other two phages G 12 and S 7 which indicate that they are not identical.

Phage K is a serologically distinct phage and is assigned to a separate group D. It is of interest since it has such a wide range of activity against both coagulase-positive and negative staphylococci. The only phage to which it bears any appreciable relation is W, which lyses only some coagulase-negative staphylococci. Phage W is assigned to a separate group E.

Phages K and W are obviously also distinct from all the other phages examined.

Group F phages are neutralized by a serum prepared against phage 1339/789 and not by the other sera. No cross-reactions occurred between this serum and the phages of the other groups. The phages in this group were isolated from lysogenic coagulase-positive staphylococci of human origin. They resemble in many respects those of group A. The phages which fall into this group are 1339/789, 1339/CK 5, 1339/145, 1339/S 17, 1339/211, 145/211, CK 5/145 and 21. None of these phages has been used in the routine typing of staphylococci. There remains one phage, S17/211, isolated from a lysogenic strain, which was not neutralized by any of the available sera.

*Diversity of serological characters of phages 42B, 42C, 42D and 42E*

Table 1 shows that phages 42B and 42E fall into serological group A, whereas 42C and 42D belong to group B. Since these phages were all developed by adaptation from a single, presumably pure phage, their history was accordingly re-examined. The original 42 filtrate was obtained by lysing a strain 34 with the phage carried by strain 36. Strain 34, however, also carried a phage which lysed 36. Phage 42A was derived from 42 by adaptation to strain 36, i.e. it was 42/36. Phage 42B was phage 42 adapted to strain 1163, and phage 42E was 42 adapted to strain 1670. However, 42C was derived from phage 42A adapted to strain 1307, and was used for adaptation to strain 1363, becoming 42D.

Strains 34 and 36 were re-examined for lysogenicity by cross-testing on agar plates with the stock propagating staphylococci. Strain 34 was found to be lysogenic for strains 3, 36, 1670 and R1760, whereas strain 36 carried phage which lysed strains 3, 34, R1760 and 1307. Phages 36/3 and 36/1307 were then isolated by picking single plaques and replating several times. Active filtrates of these phages were then tested against sera 42D and 51. Phage 36/3 was neutralized by serum 51 and not by serum 42D, whereas the reverse was the case with phage 36/1307. It is apparent, therefore, that in the process of lysis of staphylococcus 36 by phage 42A the phage carried by 36 and active against 1307 had also been set free. This phage was present in small numbers in the filtrate 42A. When the undiluted filtrate was added to strain 1307 during the process of adaptation, the plaques developing on 1307 were due to this phage. It follows that phage 42C is not a true adapted phage and is unrelated to phages 42B and 42E. It is also of interest that the staphylococcus strain 36 carries two phages which are antigenically distinct.

Phages 42 and 42A have been discarded from the collection, but phage 47 is apparently similar to them and is propagated on strain 36. The stock

preparation of 47 gave a phage count of 210,000,000 particles/ml. when plated on 36, and of 1000 particles/ml. when plated on 1807, a ratio of 1:210,000. This ratio might be considered, in the absence of serological examination of the phages, as evidence of mutation, but is, in fact, due to the contamination of the 47/36 filtrate with phage derived from 36. Since a large proportion of staphylococci are known to be lysogenic, it follows that in some cases apparent adaptation of phages may be due solely to the manifestation of contaminating phages derived from the propagating staphylococci.

*Correlation of serological groups with other characters of the phages*

Some of the characters of the phages and of the staphylococci which they lyse are set out in Table 2.

*Range of staphylococci lysed.* Group A phages lyse only coagulase-positive staphylococci of human origin, whereas those of group B, although lysing only coagulase-positive cocci, attack both human and bovine strains. The group

Table 2. *The characters of the six serological groups of staphylococcal bacteriophages and of the range of staphylococci which they lyse*

| Serological group | Characters of bacteriophages |                      |                 |                              |                                 | Characters of susceptible staphylococci |           |                          |
|-------------------|------------------------------|----------------------|-----------------|------------------------------|---------------------------------|---|-----------|--------------------------|
|                   | Multiplication at 37°        |                      | Stability at 4° | Inactivation in 1 hr. at 49° | Range of mean plaque size (mm.) | Origin                                  | Coagulase | Phage inactivating agent |
|                   | On agar                      | In Todd-Hewitt broth |                 |                              |                                 |   |           |                          |
| A                 | +                            | +                    | +               | +                            | 0.3-0.7                         | Human                                   | +         | Nucleo-protein           |
| B                 | +                            | -                    | -               | +                            | 0.3-1.2                         | Human, bovine                           | +         |                          |
| C                 | +                            | -                    | ±               | X2-<br>S7 partial<br>G12+    | 0.7-1.0                         | Ovine                                   | +         |                          |
| D                 | +                            | +                    | +               | Partial                      | 0.5                             | Human, animal                           | +         | Polysaccharide           |
| E                 | +                            | +                    | +               | -                            | 0.6                             | Human                                   | -         |                          |
| F                 | +                            | -                    | +               | +                            | 0.5                             | Human                                   | +         |                          |

C phages are of ovine origin, and their antigenic overlap with the group B phages has already been mentioned. Phage K, which lyses both coagulase-positive and negative staphylococci has an antigenic relationship only with phage W, which lyses about 80 % of the coagulase-negative staphylococci tested. There therefore appears to be a clear-cut relationship between the serological characters of the phages, their origins, and certain of the characters of the staphylococci sensitive to them.

*Plaque size.* The staphylococcal phages produce relatively small plaques on agar. Plaque size varies inversely with the concentration of agar in the medium. Brands of agar vary considerably in the consistency of the gels they produce. For example, with powdered agar of New Zealand origin, a concentration of 0.8 % gives a gel of consistency comparable to that of the 1.25 % shredded Japanese agar used in many laboratories in the past. The shred agar

proved superior to the powdered agar which, in the concentration giving maximum plaque size, has a tendency to slip around in plates and is therefore more difficult to manipulate. Provided that attention is paid to the brand of agar used and it is employed in a suitable concentration, measurements of plaque size should be replicable on a standard medium.

In the present investigation a medium containing 1.25 % of shredded agar was used, with added peptone, Yeastrel and Fildes's peptic digest of blood; and the plaques grown overnight at 37°. This medium was developed in the Staphylococcal Reference Laboratory to provide satisfactory conditions for the maximum development of plaque size.

For any given phage there is a fairly wide range of plaque size, and 20–50 plaques were measured to obtain a mean plaque size. Among group A phages it varied from 0.33 to 0.7 mm., the range of individual diameters being 0.1–1.0 mm. The group A phages are as a whole less variable than the group B phages. Among the group B phages there are some which produce plaques up to 1.7 mm. in diameter (phage 44). Though mean plaque sizes range from 0.3 to 1.2 mm., the group B phages tend to produce larger plaques than any other group except group C, the ovine phages which produce plaques up to 1.3 mm. in diameter. X2 plaques are characteristic, being large and overgrown by secondary bacterial growth, giving a hazy appearance after overnight incubation. The phages K, W and 21 produce moderately sized plaques with no particular distinguishing characteristics.

Table 3 gives the range of plaque sizes and the mean plaque sizes in mm. for the majority of the phages, and illustrates the variation in plaque size.

*Growth requirements.* Many staphylococcal phages are difficult to grow in broth cultures (Burnet & Lush, 1935; Fisk, 1942). Certain of Burnet & Lush's phages grew only on agar at 22° and not at 37°. Phage K is an outstanding exception in that it regularly clears broth cultures. In contrast to Burnet & Lush's 'weak' phages, all the phages used in this investigation produce plaques at 37° on agar cultures containing Fildes's peptic digest of blood and Yeastrel, although irregular results were obtained in broth cultures. An explanation of these irregular results must be sought in the action of some factor other than temperature.

An attempt was made to estimate the time of the first release of phage from infected cells in Todd-Hewitt broth (1932). To 0.9 ml. quantities of broth placed in tubes in a water-bath at 37°, 0.04 ml. of a young broth culture of the appropriate staphylococcus was added, followed by 0.1 ml. of phage so diluted in broth that a 0.02 ml. quantity of the final mixture on plating would give 10–20 plaques. Samples of 0.02 ml. were plated at the moment of mixing and at intervals up to 2 hr.; a sudden increase in the phage titre indicated that lysis of infected cells had occurred.

Phages K and W grew in these conditions, and after overnight incubation cleared the cultures. Among the group A phages, 3A, 3B, 3C, 14 and 51 multiplied in these conditions, the first release of phage taking place in 55–70 min. This generation time is long compared with that of *Bact. coli* phages or of staphylococcal phage with a generation time of 35 min.

The average burst size for phage 51 was found to be 85. Taking into account the long latent period and the relatively small number of phage particles released from each infected cell it is obvious why complete clearing of broth cultures infected with these phages is rarely seen; the growth of the staphylococcus quickly outstrips that of the phage unless the dose of added phage is a large one.

Table 8. *Plaque sizes of staphylococcal phages*

| Group | Phage | Plaque size (mm.) |      |
|-------|-------|-------------------|------|
|       |       | Range             | Mean |
| A     | 3B    | 0.1-0.9           | 0.6  |
|       | 3C    | 0.2-0.6           | 0.4  |
|       | 14    | 0.2-1.0           | 0.7  |
|       | 51    | 0.1-0.6           | 0.4  |
|       | 6     | 0.2-0.6           | 0.35 |
|       | 7     | 0.1-0.6           | 0.33 |
|       | 47    | 0.1-0.7           | 0.43 |
|       | 47A   | 0.4-0.8           | 0.58 |
| B     | 29    | 0.3-1.0           | 0.68 |
|       | 29A   | 0.1-0.8           | 0.5  |
|       | 31    | 0.2-1.0           | 0.7  |
|       | 31A   | 0.1-0.6           | 0.3  |
|       | 42C   | 0.5-1.2           | 0.8  |
|       | 42D   | 0.1-0.8           | 0.38 |
|       | 44    | 0.8-1.7           | 1.2  |
|       | 44A   | 0.5-0.9           | 0.6  |
|       | 52    | 0.5-1.4           | 1.0  |
| C     | 52A   | 1.0-1.5           | 1.2  |
|       | X2    | 0.7-1.3           | 1.0  |
|       | G12   | 0.3-1.0           | 0.76 |
| D     | S7    | 0.3-1.2           | 0.68 |
|       | K     | 0.3-0.7           | 0.5  |
| E     | W     | 0.4-0.7           | 0.62 |
| F     | 21    | 0.2-0.7           | 0.46 |

The results with the other group A phages in Todd-Hewitt broth were equivocal. In some cases a rise in phage count occurred after 100-110 min. incubation, but no clear-cut evidence of bursts could be obtained. However, on the addition of 0.002 mg./ml. tryptophan to the broth these phages reproduced in a normal manner.

No evidence of multiplication of group B and group C phages could be obtained. Indeed, in certain cases, notably 29, 42D, X2, G12 and S7, at the end of an hour's incubation very little of the added phage could be demonstrated on subculture. The inactivation of phages X2, G12 and S7 is almost complete under these conditions (Table 4). With the remainder of the group B phages and phage 21, there was a loss of 50-80 % of phage. That this phage inactivation was not due to the temperature of incubation was shown by the fact that no inactivation occurred in broth containing no bacteria.

Work in progress with the group B and group C phages indicates that they require the addition to the broth of factors present in the vitamin B complex

and of unknown factors that are removed from the broth during its sterilization by heat. The present interest lies in the fact that the variability in growth requirements is associated with serological character.

Table 4. *Inactivation of ovine staphylococcal bacteriophages on incubation at 37° in Todd-Hewitt broth cultures of their propagating strains*

| Time (min.) | Phage count/ml. |        |       |
|-------------|-----------------|--------|-------|
|             | X 2             | G 12   | S 7   |
| 0           | 4,000           | 30,000 | 3,100 |
| 30          | 600             | —      | —     |
| 60          | 100             | 200    | < 50  |
| 70          | 200             | 100    | < 50  |
| 80          | 100             | 50     | < 50  |
| 90          | 50              | < 50   | < 50  |
| 100         | 50              | < 50   | < 50  |
| 120         | < 50            | 50     | < 50  |

Table 5. *Inactivation of staphylococcal bacteriophages after heating for 1 hr. at 49°. The results are expressed as percentages of phage particles surviving*

| Bacteriophages                | % surviving |
|-------------------------------|-------------|
| 6, 7, 21, 42D, 47, 47 A, G 12 | 0           |
| 14, 29, 51                    | 1-5         |
| 8 A, 3 B, 3 C, K              | 20-30       |
| S 7                           | 50          |
| X 2, W                        | 90-100      |

*Stability.* It had already been found by Dr V. D. Allison (personal communication) that certain of the phages used for typing could be maintained in the refrigerator for long periods of time without any loss of activity, while others showed a marked fall in titre on testing a few weeks after preparation. But during the transport of phage filtrates from England to Australia, by air in a refrigerator, certain of the phages became inactive. These unstable phages proved to be groups B and C. Groups A and F phages and phages K and W were, on the contrary, remarkably stable.

This instability of group B phages may be an expression of their general sensitivity to a variety of agents. Thus phage 42D lost 95 % activity after 90 min. at 37° in quarter-strength Ringer's solution. Such inactivation did not occur when the phage was incubated in broth or normal serum. Other phages in group B were not so markedly unstable. Nevertheless this instability makes it necessary constantly to check group B phages used for typing.

*Effect of temperature.* The instability of the group B and C phages made it of interest to determine the temperature of inactivation in all the serological groups. An exposure of 60 min. in a water-bath at 49° was chosen. The phages were so diluted in broth that, on plating, countable numbers of plaques were obtained. Samples were plated out at 0 and 60 min. (Table 5). With two exceptions, namely, phages X 2 and W, in which the survival rate was 90-100 %, inactivation of the phages occurred. Most of group A was devoid of activity

after heating, but 20–80 % of phages 8A, 8B and 8C were viable at the end of 1 hr. Phage K resembled phages 8A, 8B and 8C in its behaviour. The different survival rates of the ovine phages reflect the differences between these three phages already indicated by the incomplete inactivation of S7 and G12 by anti-X2 serum.

One practical conclusion to be drawn from these observations is that heat treatment of lysates or of cultures of lysogenic staphylococci is of no value in freeing such material from staphylococci, since the phages are inactivated at lower temperatures than are the bacteria.

### DISCUSSION

In a previous paper (Rountree, 1947*b*) it was reported that nucleoprotein fractions from coagulase-positive strains 145, 8 and 878 inactivated phage 51. It should be noted that 145 is the propagating strain for phage 51, 8 for phage 6 and 878 for phage 44A, and that these phages belong to the serological groups A and B. These results with phage 51 were in contrast to those obtained by Freeman (1987), who isolated from strains of staphylococci a polysaccharide which, in high dilutions, inactivated phage Au2 of Burnet & Lush's series. This phage is apparently similar to if not identical with phage K (serological group D). The behaviour of phages K and 51 with regard to serology, range of organisms lysed and time of multiplication indicates clear-cut differences between these two phages. It is therefore reasonable to suppose that they may be absorbed by different components of the staphylococcal cell surface.

One consequence of the different serological behaviour of the various staphylococcal phages is the necessity of characterizing staphylococcal phages as completely as possible, whether they are to be used for phage typing of staphylococci or for observations on the fundamental problems of phage-bacterium relationships. The necessity of using antigenically similar phages for the identification of Vi strains of *Bact. typhosum* has recently been stressed by Craigie & Felix (1947). The occurrence as contaminants in stock filtrates of phages carried by the lysogenic staphylococci used in the propagation of the stock phages, adds additional weight to the necessity of serological examination of phages appearing during adaptation of phage from one strain of staphylococcus to another, and for caution in interpreting results of apparent phage mutation among the staphylococcal phages.

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My thanks are due to Dr G. S. Wilson, Director of the Public Health Laboratory Service for permission to work in the Staphylococcal Reference Laboratory, and to Dr V. D. Allison for his kindness in placing at my disposal all the facilities of the Reference Laboratory and all his extensive knowledge of the phages. I would also wish to thank Mr G. Heimer for much valuable technical assistance.

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## The Isolation in Eggs of a New Filtrable Agent which may be the cause of Bovine Lumpy Skin Disease

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**SUMMARY:** Using a filtrate from an emulsion of skin nodules, lymph node and a milk-duct nodule from a calf dead of bovine Lumpy Skin Disease as starting material, a filtrable agent was isolated by serial passage in chick embryos. The agent affects mainly the skin structures of the chick embryo and results in a characteristic shrunken featherless embryo tightly wrapped in its amnion, and with almost complete disappearance of amniotic fluid.

The agent appears to be a filtrable virus approximately 10–25  $\mu$  in diameter. It was not pathogenic for day-old chicks and adult fowls.

The virus was neutralized by the serum of fowls which had received repeated intramuscular injections of the virus, suggesting that the virus is etiologically related to Lumpy Skin Disease in cattle. Further work is needed before such a relationship can be regarded as established.

The virus withstood heating to 60° for 20 min., survived in 50 % glycerol for at least 1 week, and at a temperature of +4° for at least a month. It was destroyed by boiling for 2 min. and by incubation at 37° overnight. Desiccation destroyed a large part of the virus in a suspension, but that part which withstood the drying process survived for at least a month.

Towards the end of 1946 'Lumpy Skin Disease' in cattle became a serious problem to dairy farmers in the Western Cape Province, Union of South Africa. The disease had been recognized previously and has been referred to as pseudo-urticaria (MacDonald, 1931). It is a condition characterized by transient fever, nodular lesions in the skin and lymphadenopathy, which go on to necrosis. The necrotic areas in the skin separate, leaving superficial ulcers which ultimately heal. The mortality is low and results usually from secondary infections such as pneumonia. Animals do, however, suffer severely in general condition and milk yields are reduced. A summary of the present knowledge of the disease has been published by de Boom (1947), and the clinical picture described by Thomas & Mare (1945) and von Backstrom (1945).

Experimental work on this disease has been carried out at The Veterinary Research Institute, Onderstepoort. On the basis of the results, the histological appearances of the lesions and the epidemiology of the disease, it was suggested that the etiological agent was probably a filtrable virus. The virus had, however, not been isolated, and transmission experiments succeeded only in bovines (Thomas & Mare, 1945). By its high incidence in dairy herds in the Cape Peninsula the disease soon threatened to create serious economic and nutritional problems, and prompted us to undertake the investigation recorded in this paper.

### *Material*

It was anticipated that if the disease were caused by a virus, the infective agent would be found in highest concentration in the lesions present during the early stages of the disease. A striking early manifestation was marked

enlargement of superficial lymph nodes such as the prescapular, and lymphoid material excised during life or at autopsy was consequently used. Skin nodules which, when possible, were excised during the first few days of the disease and before the onset of gross necrosis, were also used. Not only did we anticipate a higher virus content of such early skin nodules, but the chances of secondary infection were minimized by the use of nodules with unbroken overlying epithelium.

The first attempt to isolate the virus was made with a skin nodule excised under local anaesthesia from the flank of a Jersey heifer on the second day after appearance of cutaneous nodules (series L). A second attempt was made from skin, lymph node and a mammary duct nodule from a Jersey calf which died during the first week of a severe attack of Lumpy Skin Disease. At autopsy this calf was found to have suffered also from redwater fever, which probably contributed to its death (series W). Subsequently two more attempts were made to isolate the agent from lymph nodes, excised during the early stages of the disease, and three other attempts were made with excised skin nodules as starting material.

A virus was isolated only in one series (W), reported in detail below. With the possible exception of series L all other attempts were negative in spite of two to five serial egg passages. Some of these were abandoned because of contamination. No significant lesions were encountered apart from an occasional gelatinous oedema of the chorio-allantois.

### *Methods*

The nodules were excised together with an adequate rim of normal skin. The excised skin was pinned out, the hair and superficial layers of epidermis removed with a sharp razor, and the nodules thoroughly cleaned with a swab soaked in ether. The actual nodules in the skin were then excised, minced with sterile scissors, ground up as efficiently as possible in a mortar with an excess of Pyrex glass powder, and emulsified in Hartley's broth.

Such crude emulsions were of course heavily contaminated with a variety of organisms. Before inoculation into eggs they were therefore either filtered through gradocol membranes with an average pore diameter (A.P.D.) of 600–1000  $m\mu$  or treated with penicillin. On several occasions, including series W in which a virus was isolated, streptomycin was also added.

The emulsions were used for the inoculation of rabbits by the intradermal route and of chick embryos. On two occasions mice, gerbilles and guinea-pigs were used in addition, a variety of inoculation routes (intradermal, intra-peritoneal and intranasal) being employed. Serial transfers were carried out only in eggs, usually at 8–6 days' intervals. The results obtained with laboratory rodents were uniformly negative, and these animal experiments will not be described further. Negative results with the usual laboratory animals have been reported also by the workers at Onderstepoort (cf. de Boom, 1947).

Eggs incubated for 9–14 days at 39° were inoculated with approximately 0.25 ml., half being injected into the amniotic cavity and the remainder placed on the chorio-allantoic membrane. The inoculation technique employed was

that described by Beveridge & Burnet (1946). In some cases chorio-allantoic membrane alone emulsified in Hartley's broth was used for serial transfer. In the majority of cases, however, chorio-allantois, amnion and the whole embryo except eyes, legs and wings, emulsified in approximately 15 ml. of embryonic fluids and broth, was used. In filtration experiments embryo emulsions received preliminary clarification through paper-pulp, after which they were passed through a series of gradocol membranes with A.P.D.'s varying from 1000 to 21  $m\mu$ .

## RESULTS

### *Isolation of a virus*

*Series L.* A cutaneous nodule obtained by biopsy during the second day of skin manifestation was emulsified in broth with added penicillin, and the material inoculated into four 10-day-old eggs. Two eggs opened on the 4th day after inoculation had oedematous semi-opaque chorio-allantoic membranes. One showed in addition an amniotic sac so empty of fluid that the amnion was closely applied to the embryo. Another opened on the 5th day showed a number (c. 30) of indistinct small focal lesions in the chorio-allantois.

From the first two chorio-allantoic membranes and succeeding ones a number of serial transfers were made in eggs. Most of the eggs used for these passages were 12–14 days old before inoculation. One such series went to the 7th passage, others were discontinued after 3rd, 4th or 5th passage. Attention was paid almost entirely to the focal lesions on the chorio-allantois. They occurred, however, with great irregularity, and with continued passage became increasingly difficult to identify even with the aid of a hand lens. Although this attempt to isolate a virus was finally abandoned, several observations were made which are worth recording in the light of subsequent observations.

Thus a very marked gelatinous oedema, especially of the chorio-allantois but occasionally also of the embryo itself, was noted repeatedly. In one of the eggs, which received the original skin emulsion, the amnion was tightly applied to the shrivelled embryo—an appearance recognized as significant in the next series (W); and in an egg of 5th serial passage the embryo showed abnormal feather development, with haemorrhage in the feather follicles and feather shafts.

*Series W.* The starting material consisted of an emulsion of skin nodules, lymph node and a milk-duct nodule from a calf examined within an hour of death from Lumpy Skin Disease and redwater fever. Part of the emulsion was treated with penicillin and streptomycin and the remainder was filtered through a gradocol membrane with an A.P.D. of 600  $m\mu$ . The material received no preliminary clarification other than an hour's centrifugation at c. 8000 r.p.m. in a horizontal centrifuge. A second group of eggs received the penicillin- and streptomycin-treated emulsion without preliminary filtration. This material was discarded after three serial passages because of gross contamination. All these eggs and those used for subsequent transfers had been pre-incubated for 9–11 days before inoculation.

Four of the six eggs which received the filtered material were dead on or

before the sixth day after inoculation. One of the survivors opened on the sixth day showed marked gelatinous oedema of the chorio-allantois and numerous haemorrhages into the feather follicles. Emulsions of this embryo and its membranes in Hartley's broth were bacteriologically sterile, and when injected into eggs produced the picture which subsequently came to be regarded as characteristic for this virus, and has been uniformly reproduced in all succeeding experiments, in which more than 1500 eggs have been used. The characteristic appearance has so far persisted through more than thirteen serial passages.

Eggs usually received 0.25 ml. of material. They were usually opened on the 4th or 5th day after inoculation, when affected eggs characteristically showed marked gelatinous oedema of the chorio-allantois, so that this membrane was often nearly 0.5 cm. thick, semi-opaque and jelly-like in consistency. Well-defined focal lesions could not be distinguished, although large non-specific lesions were occasionally present along the larger blood vessels.

The amnion was often thickened and more opaque than normal, but well-marked oedema like that in the chorio-allantois was seldom encountered. The amnion was usually tightly contracted round the undersized, shrivelled embryo. The embryo itself, apart from its small size, was tightly curled up and with little or no feather development. This feature varied a great deal according to the age of the embryo when infected.

Embryos more than 10 days old at the time of inoculation usually showed more feather development, especially along the back. The feathers were, however, poorly developed, the follicles and shafts irregularly swollen and often haemorrhagic. In younger eggs there was as a rule only slight follicle development limited to the back, and this part was frequently loosely adherent to the closely applied amnion. Numerous intracytoplasmic inclusion bodies were demonstrable in the skin and subcutaneous tissues. The histological features will be reported in detail elsewhere. The appearance of the featherless, shrunk, curled up embryo in its amnion was reminiscent more of an insect pupa enclosed within its pupal case than of a chicken (see Plate 1).

#### *The properties of the virus and attempts at its identification*

**Filtration.** Emulsions of whole embryo and membranes in Hartley's broth were, after preliminary clarification through paper-pulp, filtered in series through gradocol membranes with A.P.D.'s of 670, 270, 116 and 52  $\mu$ . All filtrates were free of an indicator organism, *Chromobacterium prodigiosum* (*Serratia marcescens*), with which the original emulsion had been heavily seeded. Each filtrate was used for the inoculation of six 11-day-old eggs. They were observed for 4 days, after which all survivors were opened. (Limited supplies of eggs did not allow of titration of each filtrate.) The results recorded in Table 1 show that all filtrates contained the active agent.

There was some doubt at this stage whether the agent responsible for the abnormalities in the chick embryos could be regarded as a virus; that instead it might be a soluble toxic substance produced by a contaminating organism

which would not grow in the ordinary bacteriological media in current use in the laboratory. In that event the agent would not be transmissible in series from eggs which in the first place had received a bacteria-free filtrate. Accordingly, one of the embryos (in Table 1) which showed specific lesions after inoculation with a 116 m $\mu$  filtrate was used for further filtration, through membranes with A.P.D.'s of 270, 116, 52 and 21 m $\mu$ . That a filtrate through membranes of A.P.D. 52 m $\mu$  or higher is still active is shown by the results

Table 1. *Filtration of the virus through gradocol membranes*

| A.P.D. of membranes (m $\mu$ .) |              |             |              |
|---------------------------------|--------------|-------------|--------------|
| 670                             | 270          | 116         | 52           |
| D5 Autolysed                    | D5 Autolysed | D5 Typical  | D5 Autolysed |
| D5 Autolysed                    | D5 Autolysed | D5 Typical  | D5 Autolysed |
| D5 Typical                      | D5 Typical   | K5 Typical* | D5 Typical   |
| D5 Typical                      | D5 Typical   | K5 Typical  | K5 Typical   |
| K5 Typical                      | D5 Typical   | K5 Typical  | K5 Typical   |
| K5 Typical                      | K5 Typical   | K5 Typical  | K5 Typical   |

\* Used for further filtration (see text p. 178 and Table 2).

D5=died on or before 5th day.

K5=alive and opened on the 5th day.

recorded in Table 2. The filtrate obtained through a membrane with A.P.D. of 21 m $\mu$ , on the other hand, completely withheld the virus. Although the exact determination of size requires further investigation, these results suggest that the particle size of the active agent lies between 10 and 25 m $\mu$ . Repetition of these experiments gave similar results.

Table 2. *Filtration of the virus from embryo inoculated with a filtrate (cf. Table 1) through gradocol membranes*

| A.P.D. of membrane (m $\mu$ ) |            |            |               |               |
|-------------------------------|------------|------------|---------------|---------------|
| 270                           | 52         | 52         | 21            | 21            |
| Dilution of virus             |            |            |               |               |
|                               | 1/1        | 1/100      | 1/1           | 1/100         |
| D5 Typical                    | D5 Typical | D5 Typical | K5 No lesions | D5 Autolysed  |
| K5 Typical                    | K5 Typical | D5 Typical | K5 No lesions | K5 No lesions |
| K5 Typical                    | K5 Typical | K5 Typical | K5 No lesions | K5 No lesions |
| K5 Typical                    | K5 Typical | K5 Typical | K5 No lesions | K5 No lesions |
|                               |            |            | K5 No lesions | K5 No lesions |
|                               |            |            | K5 No lesions | K5 No lesions |

D5=died on or before 5th day.

K5=alive and opened on the 5th day.

*Titres of the virus in the chick embryo.* Emulsions of whole embryo and membranes in a total volume of 15 ml. of mixed embryonic fluids and broth, centrifuged for 20 min. at c. 3000 r.p.m., have been repeatedly titrated. Tenfold dilutions of the emulsion in Hartley's broth were each used to inoculate groups of three to six eggs. The results of a typical experiment are recorded in Table 3.

Because non-specific deaths amongst the chick embryos were fairly frequent and irregular, reliance has not been placed on eggs which were dead at the time of opening (3rd, 4th or 5th day) unless autolytic changes were so slight that the lesions were unmistakable. Several times the results with higher dilutions were irregular, but even if that dilution giving rise to characteristic

Table 3. *Result of titration of virus suspension in eggs*

| Dilution of virus suspension |                  |                  |                  |                  |
|------------------------------|------------------|------------------|------------------|------------------|
| 10 <sup>-2</sup>             | 10 <sup>-5</sup> | 10 <sup>-6</sup> | 10 <sup>-6</sup> | 10 <sup>-7</sup> |
| 2D4 Autolysed                | 2D3 Autolysed    | —                | 3D3 ?NS          | —                |
| 2K4 Typical                  | 2K4 Typical      | 5K4 Typical      | 1D4 Autolysed    | 6K4 No lesions   |
| —                            | —                | —                | 1K4 Typical      | —                |

?NS= probably non-specific.

*x*D*y*=*x* dead on or before the *y*th day.

*x*K*y*=*x* alive and opened on the *y*th day.

lesions in all the eggs inoculated, and which survive to the 4th day, be defined as the end-point, the titre must be accepted as fairly high: at least 10<sup>-5</sup> of an emulsion consisting of one embryo in 15 ml. Subsequent titrations indicated an even higher virus content. Thus in neutralization tests reported below 10 % suspensions (w/v) of virus-infected embryos in broth were used. These suspensions on several occasions attained a titre of 10<sup>-8</sup>.

*The effect of drying.* Emulsions of whole embryo in allantoic fluid, centrifuged for 20 min. at *c.* 3000 r.p.m., were distributed in ampoules in 0.5 ml. amounts and then dried in a high-vacuum spin freeze-drier (Greaves, 1944), with and without preliminary freezing. The titre of reconstituted dry suspension has on each occasion been found to be approximately 10<sup>-2</sup>. There is therefore at least a thousandfold drop in titre on drying by this method, but once dry the material maintained its potency with little or no alteration for several months.

Table 4. *Susceptibility of the virus to heat*

| Treatment            |                      |                 |
|----------------------|----------------------|-----------------|
| Unheated             | 60° for 20 min.      | 100° for 2 min. |
| 1D4 Typical          | 1D4 Autolysed        | 4K4 No lesions  |
| 4K4 Typical          | 2K4 Typical          | —               |
| 1K4 Typical (slight) | 1K4 Typical (slight) | —               |

*x*D*y*=*x* dead on or before the *y*th day.

*x*K*y*=*x* alive and opened on the *y*th day.

*Susceptibility of the virus to heat.* Samples of undiluted emulsions of virus-infected embryo were heated to 60° for 20 min. or to 100° for 2 min. The heated emulsion, as well as control material which had been left at room temperature, was inoculated into 10-day-old chick embryos. The results recorded in Table 4 show that this virus can withstand heating to 60° for 20 min., but that boiling destroys it. In one experiment it was found to be destroyed by overnight incubation at 87°. The virus maintained its infectivity for the eggs at refrigerator

temperatures (+4°) for at least a month, and its potency for at least a week in 50 % glycerol at +4°.

*Pathogenicity for chicks and adult fowls and preparation of immune serum.* Adult fowls and day-old chicks were obtained from the supplier of the fertile eggs used in this investigation. Fresh chick embryo emulsion was injected intramuscularly and subcutaneously into four adult fowls and four 1-day-old chicks. At the same time some of the inoculum was rubbed into the skin of a small area on the chest from which the down or feathers had been plucked. None of the birds developed any symptoms or signs of illness during a 3 weeks' observation period.

Table 5. *Neutralization of virus by immune fowl serum, and by normal and convalescent bovine sera*

|   |                      | Inoculum                  |                           |                                       |  |
|---|----------------------|---------------------------|---------------------------|---------------------------------------|--|
|   | Virus + broth        | Virus + normal fowl serum | Virus + immune fowl serum | Virus + 'normal' bovine serum (local) | Virus + convalescent bovine serum (6440) |
| A | 2D4 Autolysed        | 1D4 Autolysed             | 1D4 Autolysed             | —                                     | 2D4 Autolysed                            |
|   | —                    | 1K4 No lesions*           | —                         | —                                     | —  |
|   | 3K4 Typical          | 3K4 Typical               | 3K4 No lesions            | —                                     | 2K4 No lesions                           |
| B | 2D4 Autolysed        | —                         | —                         | 2D4 Autolysed                         | 2D4 Autolysed                            |
|   | 4K4 Typical          | —                         | —                         | 1K4 Typical                           | 1K4 Slight lesions                       |
|   | 1K4 Typical (slight) | —                         | —                         | 3K4 Slight lesions                    | 4K4 No lesions                           |
|   | —                    | —                         | —                         | 1K4 No lesions                        | —  |

\* The absence of lesions in one egg while others of the same group showed typical lesions, was seen very occasionally even using inocula of many infective doses. The explanation is that occasionally the inoculum is accidentally placed outside the amniotic cavity, in spite of precautions taken to avoid this.

$x\text{Dy} = x$  dead on or before the  $y$ th day.

$x\text{Ky} = x$  alive and opened on the  $y$ th day.

The adult fowls then received six further injections at 2- or 3-day intervals, each of one-quarter of a virus-infected embryo and membranes emulsified in saline. They were bled from a wing vein on the 10th day after the last injection, and the Seitz-filtered serum was stored in measured amounts in the dry state.

*Neutralization tests with the sera of fowls immunized against the egg virus and of bovines convalescent from Lumpy Skin Disease*

In the earlier part of this work dry stock virus of known titre was used. The dry stock virus with a titre of  $10^{-3}$  was reconstituted in broth. Appropriate dilutions were mixed with equal volumes of various serum samples under test. In the controls broth was used instead of serum. The results of a typical experiment are recorded in Table 5.

These results were obtained with relatively small numbers of eggs, but similar findings were obtained in other experiments. Table 5 shows, in addition,

that 'normal' bovine serum obtained from a local slaughterhouse possessed neutralizing power, though less than that of known convalescent sera. A study of the incidence of the disease in dairy herds revealed that often the majority of animals in a herd are refractory to the disease. This may be because these animals have suffered an inapparent infection at the time of the outbreak or previously. As the slaughterhouse was supplied from areas in which the disease was at the time prevalent, the serum obtained from them could not be regarded as providing satisfactory normal controls.

Sera were subsequently obtained from bovines which had at no time been exposed to the risk of infection, and from animals which had suffered the disease under controlled experimental conditions. These sera were tested according to the method here described, as well as by a second method using fresh virus suspensions instead of dried virus. The modification in technique was introduced because of the possible interference with the neutralizing power of serum by a large amount of inactivated virus present in dried suspensions. With one exception the results of these further experiments confirmed the presence of neutralizing antibody in the sera of cattle convalescent from the disease, and the absence of antibodies from the sera of normal cattle. These experiments, which were undertaken together with Dr R. Alexander of Onderstepoort, will be reported in detail elsewhere.

#### *The control serial passage of normal chick embryo material*

Throughout the preliminary work it was realized that the virus which had been isolated might bear no relation to the etiology of Lumpy Skin Disease in cattle. One of the possibilities, though extremely remote, was that serial transfer may have revealed a latent virus present in the eggs. Beveridge & Burnet (1946) point out that the usefulness of eggs in virus research depends to a large extent on the fact that no latent viruses have yet been demonstrated in eggs.

It was decided, nevertheless, to carry out a parallel series of egg passages with normal embryo as starting material. An attempt was made to carry out these passages and the virus transfer under identical conditions. Often, therefore, the virus and control passages were done at the same bench, on the same morning, and using the same instruments. It was also not realized at this time that the virus was relatively heat-resistant. Risks of accidental transfer of the virus to the control normal embryo passage series were therefore great, and this was brought out by subsequent experience. Characteristic virus lesions suddenly appeared in the control series after three passages.

A fresh series of controls was therefore started and carried out with every precaution to prevent contamination. This series was abandoned after the 10th serial transfer, without the occurrence of any lesions resembling those due to virus. Subsequently serial passages in eggs have been undertaken on numerous occasions without the appearance of lesions resembling those described above.



## DISCUSSION

There can be little doubt that the agent isolated in eggs is a virus. It produces characteristic lesions even when given in small doses, multiplies actively in the eggs, is filtrable, and is antigenic. Its size according to the preliminary experiments recorded in this paper is in the region of 10–25 m $\mu$ .

It seems probable that the occasional occurrence of gelatinous chorio-allantoic membranes and abnormalities of the embryo recorded with the first series (L) was due to the same agent. That these findings were not more frequent can probably be explained by the fact that older embryos were used for those experiments. Subsequent experience has shown that embryos are much less susceptible when infected after skin and feather development is well advanced. Also in series L attention was paid particularly to the lesions on the chorio-allantois for which the older eggs appeared at the time to be more satisfactory. Later experience with series W indicates that although virus multiplies in the chorio-allantois, it produces no constant recognizable abnormality of the membranes or embryo, apart from the somewhat irregular development of a striking gelatinous oedema of the chorio-allantois.

The exact nature of the virus cannot be established until pathogenicity tests have been carried out in a variety of animals, including bovines. The neutralization of virus by sera from animals convalescent from Lumpy Skin Disease is, however, presumptive evidence that the virus may be etiologically related to the bovine disease. It is interesting in this connexion that the apparent size of the virus is nearer that of foot and mouth disease than that of the animal pox viruses.

It is striking that isolation of the virus succeeded only on one occasion; in an earlier attempt the virus lesions were probably present, though they were unrecognized. In spite of repeated efforts, every subsequent attempt to isolate the virus has failed. It may be significant that the successful material was obtained from a fatal case in a calf during the earlier phases of the epidemic, whereas subsequent material was obtained by biopsy from adult animals, all of which recovered.

We are grateful to Dr M. Horwitz for help in collecting material; to Dr Bull, Mr H. Woods, Mr Louw, Mr H. Levinkind, Mrs Machanick and others who gave us access to material on their farms. Dr G. Selzer kindly examined the histological preparations for us, Mr W. Taylor prepared the photograph, and Mr G. S. Turner gave us valuable assistance in the laboratory. The constant helpful interest of Dr R. Alexander was a great encouragement to us.

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Normal

With virus

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#### EXPLANATION OF PLATE

The five embryos at the right show the typical lesions caused by the filterable agent. The five embryos at the left are normal, and of the same age.

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## A Method of Estimating Streptomycin in Serum and Other Body Fluids by Diffusion through Agar Enclosed in Glass Tubes

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**SUMMARY:** Streptomycin may be estimated in body fluids by allowing the fluid to diffuse through nutrient agar seeded with a culture of *Staphylococcus aureus* contained in small-diameter (3 mm. internal) glass tubes. The zone of inhibition produced is affected neither by the anaerobic conditions within the agar nor by the volume of the test fluid. Acidity of the test fluid invalidates the results, since the depth of the zone of inhibition is decreased. Increase in the size of the inoculum of the test organism also decreases the depth of the zone of inhibition. Small errors arise from variations in timing during the setting-up of the test. The zones produced in the presence of different human sera differ slightly and to a degree similar to that found in the presence of various urines.

The available mathematical expressions suggest that the square of the depth of the zone of inhibition is linearly related to the log of the concentration of an antibiotic in the test fluid. Experimentally this relationship does not hold for low concentrations of streptomycin, probably due to the assumption of boundary conditions which cannot be defined with certainty; but it is a better approximation than the assumption of a linear relationship between the depth of the zone of inhibition (unsquared) and the log of the streptomycin concentration. A method of statistical analysis is given in which a weighted regression line is fitted to the squared values of the zones of inhibition in a manner analogous to probit analysis. The routine method may be refined without undue labour to the point where assays are accurate to within  $\pm 5\%$ .

One of the most widely used methods for estimating streptomycin in body fluids is that of Stebbins & Robinson (1945). In this method the sensitivity to streptomycin of the test organism, a strain of *Staphylococcus aureus*, is increased by using 1% agar, slightly alkaline and with a low salt content. The solutions are applied to the surface of seeded plates in Oxford cylinders.

A twofold increase in the concentration of streptomycin in the fluid to be assayed produces a smaller increase in the diameter of the zone of inhibition than is produced if similar solutions of penicillin are used. Variations caused by the method of applying the solution to the seeded agar therefore diminish the accuracy of the estimation to a greater extent. When using conventional plate methods with Oxford cylinders, filter-paper disks or cups cut with a cork borer, this source of variation decreases the accuracy of the estimation considerably. Variation is also increased by failing to use plates of uniform size with flat bottoms, which are difficult to obtain at the present time. It has been shown by Hayes (1945) that the depth of agar in the plates affects the size of the zone of inhibition when estimating the concentration of penicillin in fluids.

To minimize these variations a method has been evolved in which the seeded agar is pipetted into lengths of 3 mm. internal diameter glass tubing

sealed at one end. The test fluid is then pipetted on to the top of the agar column thus formed. The procedure is more rapid than any of the plate methods and also allows for a greater flexibility of experimental design. It uses a minimum of apparatus and a measurement may be made on as little as 4 drops of serum. Under routine laboratory conditions nineteen out of twenty assays will lie within about 25 % of the true value. Examples of an accurate assay procedure will be given where nineteen out of twenty assays would fall within the limits of  $\pm 5$  %. Originally developed for use with serum as the test fluid, it can be used with the same accuracy for assaying streptomycin in cerebrospinal fluid, pleural, peritoneal and pericardial fluid and urine. With less accuracy it can be used for neutral or alkaline pus and for acid pus containing large amounts of streptomycin. It cannot be used for acid pus and most sputa containing small amounts of streptomycin. The fluids do not need to be sterile, nor do they need inactivation. The range is 1–1000  $\mu\text{g. streptomycin base/ml.}$ , with greatest accuracy in the range of 2–100  $\mu\text{g./ml.}$

#### THE ASSAY TECHNIQUE

*Materials used.* The test organism was *Staphylococcus aureus*, N.C.T.C. 7361, obtained originally from the Mayo Clinic where it is used for estimating streptomycin. The *Staph. aureus* Oxford H was also used and similar results obtained. Members of the genus *Bacillus* or one of the Gram-negative bacilli were not used because they were inhibited by fresh serum, but, under circumstances where this did not apply, they could be used in essentially the same manner. The *Staph. aureus* chosen was maintained by daily transfer in broth (peptone, 1 %; Lab Lemco (Oxo Ltd., London), 1 %; sodium chloride, 0.5 %), but it was possible to use the same broth culture incubated for 18 hr. and then kept in the refrigerator for a week.

The agar medium used in the assay was prepared from nutrient 2 % agar (digest broth containing 2 % Japanese agar) by diluting one volume with an equal volume of 1 % peptone and adjusting the pH to 7.8–8.0. When this agar was diluted to 0.67 % and sloped in  $6 \times \frac{5}{8}$  in. tubes the slopes retained their shape after 18 hr. incubation; with agar diluted to 0.44 % they collapsed. The nutrient 1 % agar was autoclaved in 19 ml. amounts in 1 oz. screw-capped bottles. Nutrient agar of the same gelling power, made with New Zealand agar, was less satisfactory because it was more difficult to pipette and the zones of inhibition were smaller.

Glass tubing of 3 mm. internal diameter was cut into 7–8 cm. lengths. These were sealed at one end and dry sterilized in bulk. When the tubing was narrower filling became difficult, whereas when it was wider the quantity of fluid required for the test was increased. After use the tubes were cleaned without individual handling by boiling in soap or Kinray powder solution (Reddish Chemical Works, Reddish, Stockport) followed by boiling first in dilute hydrochloric acid and then in distilled water. A microscope with a vernier millimetre scale attached to the mechanical stage and a cross-wire in the eyepiece was used for reading the depth of the inhibition zone.

### Method

(1) The nutrient 1% agar was thoroughly melted in a screw-capped container and transferred to a 45° water-bath. It was often convenient to leave it in a 56° water-bath and then transfer it for a few minutes before use to a 45° water-bath on the bench.

(2) The fluids to be tested were prepared and controls made up. Serum and cerebrospinal fluid needed no preparation. Urine was adjusted to pH 7·8, using cresol red in aqueous solution as an internal indicator, and then diluted 1 in 10 or 1 in 100 in M/15 phosphate buffer (pH 7·8), since urine usually contains a high streptomycin concentration. For the controls two different streptomycin concentrations were used, values being chosen so that one was at the upper limit and one at the lower limit of the expected range of the 'unknowns'. Thus in measuring the amount of streptomycin present in the serum of patients receiving 0·5 g. streptomycin intramuscularly every 6 hr., the amounts present in the serum ranged from about 6 to 80 µg./ml. Controls of 4 and 32 µg./ml. were therefore suitable. When the range of values to be tested was unknown, controls at 4 and 64 µg./ml. were made up. When testing serum the controls were made up in human serum. When testing cerebrospinal fluids they were made up in distilled water. Urine standards were made up in the phosphate buffer used for dilution.

(3) A roll of plasticine (Harbutt's Plasticine Works, Bath) was placed on the bench and into it were inserted vertically groups of tubes, a group for each specimen to be tested. Each of the groups for an unknown specimen contained four tubes and each for a control six tubes.

(4) From an overnight culture of the *Staph. aureus* grown at 37°, 0·1 ml. was taken and added to 10 ml. sterile water; 1 ml. of this was added to the melted agar (final dilution of 1 in 2000). With a smaller inoculum the edge of the zone of inhibition became difficult to define, and with a larger one the sensitivity of the test was decreased.

(5) The agar was then shaken vigorously to mix in the inoculum thoroughly and, with a wide-bored Pasteur pipette the seeded agar was pipetted into all the tubes as rapidly as possible. The columns of agar in the tubes were about 2–3 cm. long. As the pipette was withdrawn, a little agar was sucked back to ensure an evenly shaped meniscus.

(6) The agar was allowed to set for at least 5 min.; a shorter interval allows fluid to enter between the agar and the wall of the tube, causing an abnormally large zone of inhibition in a proportion of the tubes. The control and test fluids were then gently pipetted on to the tops of the agar columns. The volume added was immaterial so long as it produced a layer at least 1 mm. deep.

(7) The tubes were incubated overnight at 37° in a labelled rack. The depth of zone of inhibition was not altered by incubation for a further 48 hr.

(8) The depth of the zone of inhibition was read by using the millimetre scale on the traversing mechanical stage of a microscope. A small ridge of plasticine was put on each end of a microscope slide which was placed on the

mechanical stage. The tubes were laid across the two ridges. The eyepiece cross-wire was focused on the centre of the meniscus between fluid and agar using a  $\frac{3}{8}$  in. objective and reduced lighting. The readings on the scale were taken to the nearest 0.1 mm. The stage was traversed till the cross-wire was at the edge of the growth. The difference between the two readings was the depth of the zone of inhibition ( $y$ ). As one passes towards the test fluid there is first a zone of normal growth with well-spaced colonies. There is then a zone in which the colonies are of increased size and which ends abruptly. Measurements were taken from this latter edge. Since these larger colonies are as numerous as in the zone of normal growth, and since the zone has an appreciable width, it would seem unlikely that the increased size is due solely to a diminished competition for living space. As one proceeds farther towards the test fluid, one can still see a few very small colonies; their numbers gradually decrease. The sharp definition of the edge thus appears to be due to some factor causing an increase in colony size rather than to bacteriostasis alone. The decreased size of the boundary colonies was always relatively larger when measurements were made on serum than when cerebrospinal fluid or buffer solutions were used. When acid solutions containing streptomycin in low concentrations were used the zone of growth lacked this sharp edge and, instead, the number of colonies gradually decreased over a distance of 0.2–0.3 mm. or more. When this occurred the inhibition zones were always smaller than with an alkaline solution of the same concentration. Penicillin, extreme degrees of acidity or alkalinity, phenol, ethanol and acetone all tended to produce zones of inhibition of the latter type, and their presence in the fluid to be assayed could thus be recognized.

(9) The measurements for each tube having been made, the average for each group of tubes was taken and this figure squared. A graph was prepared with the square of the average zone of inhibition as the ordinate and the  $\log_{10}$  of the streptomycin concentration as the abscissa. The two control points on this graph were plotted and joined with a straight line. This line was used for reading off the  $\log_{10}$  of the streptomycin concentration in the unknown fluid. The actual value was obtained by conversion using an antilog table. A typical example is given in Table 1.

#### *Criteria for the method and notes on sources of error*

*Effect of anaerobic conditions in the agar columns.* One of the first criticisms of such a method that would be made is that anaerobic conditions are established in the depths of agar columns, and that it has been reported (Geiger, Green & Waksman, 1946; May, Voureka & Fleming, 1947) that anaerobiosis affects the minimum concentration at which streptomycin inhibits the growth of organisms. Thus it would seem to follow that a long column of fluid above the agar column would increase the degree of anaerobiosis in the top layers of the agar and that the level at which growth occurred would be altered. This was investigated by two experiments. In the first of these (see Table 2), streptomycin was incorporated in assay agar to a concentration of



10  $\mu\text{g./ml.}$  This agar was pipetted on to the top of seeded assay agar in tubes in three groups in which the length of the superposed column of agar containing streptomycin varied. There is no significant difference in the depth of the zones of inhibition caused by the three different column lengths. A similar experiment in which columns of fluid of differing length were similarly added also failed to show significant differences in the depths of the zones of inhibition.

Table 1. *Example of the method and assay of streptomycin by measuring the depth of the inhibition zone in columns of inoculated agar medium*

|  | Depth of zone of inhibition<br>(mm.) | Average<br>$\bar{y}$ | Average<br>$\bar{y}^2$ |
|--|--------------------------------------|----------------------|------------------------|
| Control 4 $\mu\text{g./ml.}$                               | 3.7, 3.7, 3.4, 3.6, 3.5, 3.8         | 3.617                | 13.10                  |
| Control 64 $\mu\text{g./ml.}$                              | 7.1, 7.4, 7.1, 7.0, 7.2, 7.0         | 7.133                | 50.90                  |
| Test fluid   | 5.6, 5.3, 5.4, 5.4                   | 5.425                | 29.43                  |
| $\text{Log}_{10}$ of streptomycin concentration from graph | [1.122]                              |                      |                        |
| Concentration of streptomycin ( $\mu\text{g./ml.}$ )       | [13.3]                               |                      |                        |

Table 2. *The effect of anaerobic conditions on the zone of inhibition. Each group contained six tubes*

| Group | Average length of agar column containing streptomycin (mm.) | Average depth of zone of inhibition (mm.) |
|-------|---|---|
| 1     | 42.5  | 6.15                                      |
| 2     | 7.3   | 6.13                                      |
| 3     | 2.6   | 6.20                                      |

| Analysis of variance |                    |             |                |         |
|----------------------|--------------------|-------------|----------------|---------|
|                      | Degrees of freedom | Mean square | Variance ratio | $P$     |
| Between groups       | 2                  | 0.00720     | 1.22           | $> 0.2$ |
| Error                | 15                 | 0.00589     | —              | —       |

In the second experiment assay agar was seeded with *Staph. aureus* and then divided into a series of portions to each of which was added streptomycin to make a series of twofold dilutions from 4 to 0.03  $\mu\text{g./ml.}$  From each, agar was pipetted into tubes to form column lengths of about 45 mm. These were incubated for 18 hr. at 37°. In the tubes containing 0.06  $\mu\text{g. streptomycin/ml.}$  or less there were numerous colonies throughout the column. In the tube containing 0.125  $\mu\text{g. streptomycin/ml.}$  and in those with higher concentrations there were very scanty colonies. In all tubes the number of colonies was approximately even throughout the column. Thus the end-point at which the majority of the cocci failed to grow was not altered by the change from aerobic to anaerobic growth that occurred over the length of the agar column.

*Effect of the pH of the fluid to be assayed.* Waksman, Bugie & Schatz (1944) and others have reported that the activity of streptomycin is diminished in acid solutions. It might thus be expected that the zones of inhibition would be diminished when acid fluids containing low concentrations of streptomycin are assayed. To investigate this, concentrations of streptomycin of 5 and 1  $\mu\text{g./ml.}$

were made up in  $M/15$  phosphate buffer at a range of pH values from 5.8 to 10, and concentrations of  $100\ \mu\text{g. streptomycin/ml.}$  were made up in  $M/5$  phosphate buffer over the same range. The results are plotted in Fig. 1. It is clear that all fluids to be assayed should thus have a pH value of 7.8 or more. This is of importance in measuring streptomycin in urine, pus or sputum, any of which may be acid.

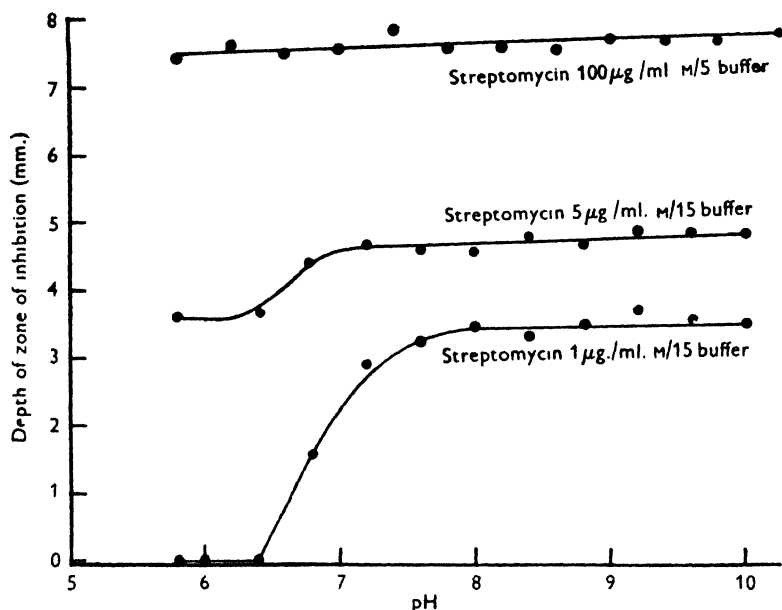


Fig. 1. Effect of pH on the zone of inhibition.

*Effect of inoculum.* An 18 hr. growth of *Staph. aureus* was added to batches of assay agar to make final dilutions of  $1/4000$ ,  $1/2000$ ,  $1/1000$ ,  $1/500$ ,  $1/250$ , and from each batch tubes were set up.  $M/10$  phosphate buffer (pH 7.8) containing 4, 40 and  $200\ \mu\text{g. streptomycin/ml.}$  was added above the agar columns. The timing of the setting up of the different batches was kept as similar as possible. The results are shown in Fig. 2. It will be seen that the smaller the inoculum, the steeper is the slope of the line relating length of zone of inhibition to  $\log_{10}$  concentration of streptomycin. Thus, to increase the sensitivity of the method, the inoculum should be as small as is consistent with accurate determination of the edge of the zone of growth.

Standard curves prepared from different tubes of seeded agar even under apparently identical conditions were found to differ from each other both in height and slope, sometimes very markedly. Probably a considerable part of this variation was due to small alterations in the size of the inoculum though there might also have been other factors. The tubes for controls and unknowns to be tested against them were always set up from the same tube of agar. Breach of this rule introduced very large errors.

*Errors in timing.* When a row of tubes was filled there was a difference between the tubes inasmuch as the first tube was filled a few minutes before

the last. This introduced a further possible source of error. If the processes of filling with agar and adding the fluid to be assayed were carried out as rapidly as possible the errors involved were very small and could be ignored in routine examinations. Filling with agar was completed in 2-3 min. or less, and adding the fluid to be assayed in 10 min. or less. For more accurate work the effect

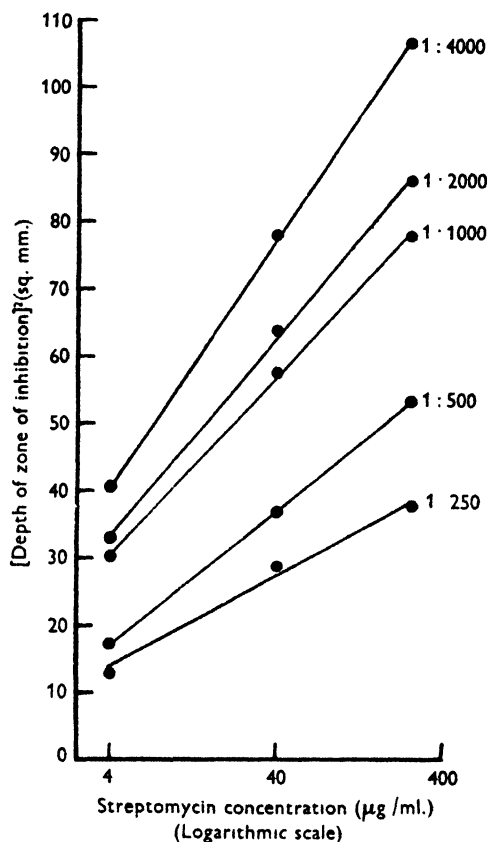


Fig. 2. Effect of inoculum on the zone of inhibition. Final dilutions of an 18 hr. broth culture from 1:250 to 1:4000.

of the timing errors could not be ignored. It was investigated in conjunction with the construction of curves relating zone of inhibition to concentration of streptomycin in the fluid to be assayed.

In considering the timing errors there were two possible sources of uncontrolled variation. One of these was in the adding of the agar to the tubes and the other was in the adding of the fluid above the agar. Restraints could be applied to these two variables and, at the same time, the relation between zone of inhibition and streptomycin concentration could be investigated by the use of a Latin square experiment. A typical example is given in Table 3. Agar was added to the tubes in row 1 first, then row 2, etc. The fluids were added above the agar in column 1 first, then in column 2, etc. The total time taken

to add the agar was 5 min. and to add the fluid 12.5 min. There were two tubes in each 'plot'. In this case streptomycin was made up in M/15 phosphate buffer (pH 7.8) in concentrations of 256, 64, 16, 4 and 1  $\mu\text{g./ml.}$  The concentration used is indicated in the corner of each plot.

Table 3. *Latin square experiment; the effect of timing errors and different concentrations of streptomycin on the depth (mm.) of the zone of inhibition*

|       |     | Columns |     |     |     |     |
|-------|-----|---------|-----|-----|-----|-----|
|       |     | 1       | 2   | 3   | 4   | 5   |
| 1     | 64  | 6.7     | 3.7 | 7.5 | 5.4 | 0.7 |
|       |     | 6.7     | 3.7 | 7.8 | 5.6 | 0.9 |
| 2     | 256 | 7.9     | 6.8 | 5.4 | 1.5 | 3.2 |
|       |     | 7.9     | 6.9 | 5.3 | 1.1 | 3.5 |
| Row 3 | 4   | 3.6     | 7.9 | 1.0 | 6.5 | 5.1 |
|       |     | 3.9     | 8.6 | 1.1 | 6.8 | 5.1 |
| 4     | 1   | 0.7     | 5.3 | 6.7 | 3.8 | 7.6 |
|       |     | 0.9     | 5.2 | 6.8 | 3.7 | 7.7 |
| 5     | 16  | 5.3     | 0.9 | 3.6 | 7.3 | 6.6 |
|       |     | 5.4     | 0.8 | 3.6 | 7.7 | 6.6 |

Concentration of streptomycin ( $\mu\text{g./ml.}$ ) in top left corners.

Table 4. *Effect of timing errors in six Latin square experiments*

| Latin square   | Range of streptomycin concentrations ( $\mu\text{g./ml.}$ ) | Approximate time (min.) taken in |              | Probability of occurrence by chance Variability due to |              |
|----------------|---|----------------------------------|--------------|--|--------------|
|                |   | Adding agar                      | Adding fluid | Adding agar  | Adding fluid |
|                |   |                                  |              |  |              |
| 6 $\times$ 6   | 2-512   | 6                                | 18           | 0.05-0.01  | 0.2-0.05     |
| 5 $\times$ 5   | 1-256   | 5                                | 12.5         | > 0.2  | 0.01-0.09    |
| 4 $\times$ 4   | 4-5   | 4                                | 8            | 0.05-0.01  | > 0.2        |
| 6 $\times$ 6   | 3-5   | 6                                | 18           | > 0.2  | 0.2-0.05     |
| 6 $\times$ 6   | 3-5   | 6                                | 18           | 0.01-0.001   | 0.05-0.01    |
| 12 $\times$ 12 | 0.8-100   | 15                               | 40           | < 0.001  | < 0.001      |

From the analysis of variance (see below), it was concluded that the timing error, due to the adding of the agar, was of no significance ( $P=0.2$ ), whereas that due to the addition of the fluid above the agar was significant ( $P=0.05-0.01$ ). An analysis was made of two similar experiments, one with streptomycin in M/15 buffer (pH 7.8) and the other with streptomycin in serum. Three similar lay-outs were used for two point assays, one a 4  $\times$  4 Latin square, the other two being 6  $\times$  6 squares. A sixth experiment using a 12  $\times$  12 Latin square was more complicated and involved longer times in setting up. The probabilities that the timing errors would occur by chance are given in Table 4. It thus

becomes apparent that both timing errors, though small, were usually significant. The relation between the depth of the zone of inhibition and the streptomycin concentration in the fluid to be assayed was also investigated in the experiment given in Table 3. It is discussed theoretically in the appendix together with a statistical analysis of the experimental results.

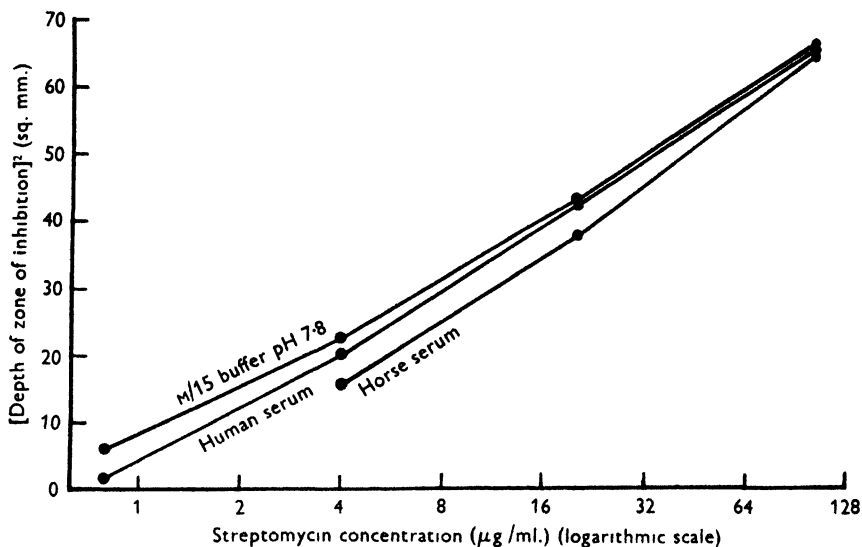


Fig. 3. Curves relating the zone of inhibition to the streptomycin concentration in the assay fluid. Comparison between M/15-phosphate buffer pH 7.8, human serum and horse serum.

*The influence of the fluid containing streptomycin.* The necessity of using human serum for the controls when estimating serum streptomycin was investigated by making up four concentrations of streptomycin in pooled human serum, in horse serum and in M/15 phosphate buffer (pH 7.8) and comparing them in a  $12 \times 12$  Latin square, using the rows and columns to restrain the two timing errors. The buffer solution gave a curve (Fig. 3) closer to the one derived from the horse serum. Even so there was a highly significant difference between the slope and the height of the curves for human serum and buffer. (Between means  $t=44.8$ ; between regressions  $t=9.39$ . For both  $P < 0.001$ .)

To determine whether all human sera containing the same concentrations of streptomycin give the same zones of inhibition streptomycin was added to twenty different sera to make a final concentration of  $10 \mu\text{g./ml.}$  Each serum was added to six tubes. The tubes were arranged in two sets of fifteen balanced incomplete blocks in which both timing errors were incorporated in the inter-block differences. The average zones of inhibition produced after adjustment to remove the interblock differences are shown in Table 5. As will be seen from the analysis of variance the differences between the zones produced by different sera were small but real. They should be taken into account in estimating the

error of the methods when the control serum is different from the test serum. The mean square for 'between sera' was approximately three times the error mean square. The error of  $x_3$ , the unknown serum derived from equation (15) in the appendix, should therefore be multiplied by  $\sqrt{3}$  or 1.73. An analysis on similar lines for urines indicated an almost identical amount of variation.

Table 5. *Zones of inhibition with twenty different sera*

| Series 1          |                     | Series 2    |                     |
|-------------------|---------------------|-------------|---------------------|
| Serum             | Zone depth<br>(mm.) | Serum       | Zone depth<br>(mm.) |
| 1                 | 5.47                | 11          | 5.12                |
| 2                 | 5.56                | 12          | 5.00                |
| 3                 | 5.53                | 13          | 5.00                |
| 4                 | 5.62                | 14          | 4.97                |
| 5                 | 5.59                | 15          | 5.01                |
| 6                 | 5.25                | 16          | 5.05                |
| 7                 | 5.48                | 17          | 4.86                |
| 8                 | 5.51                | 18          | 5.09                |
| 9                 | 5.54                | 19          | 4.96                |
| 10                | 5.53                | 20          | 4.96                |
| S.E. of each mean |                     | $\pm 0.042$ |                     |

| Analysis of variance |                       |                |                   |           |
|----------------------|-----------------------|----------------|-------------------|-----------|
|                      | Degrees of<br>freedom | Mean<br>square | Variance<br>ratio | P         |
| Series 1:            |                       |                |                   |           |
| Between blocks       | 14                    | 0.03852        | 2.23              | 0.05-0.01 |
| Between sera         | 9                     | 0.05331        | 3.08              | 0.05-0.01 |
| Error                | 36                    | 0.0173         | —                 | —         |
| Series 2:            |                       |                |                   |           |
| Between blocks       | 14                    | 0.01786        | 2.019             | 0.02      |
| Between sera         | 9                     | 0.02796        | 3.161             | 0.01      |
| Error                | 36                    | 0.00884        | —                 | —         |

#### *Notes on an accurate assay procedure*

The fluid containing the unknown concentration of streptomycin was assayed approximately so that its value was known within  $\pm 25\%$  of the true figure. The standard was made up in a concentration of  $4 \mu\text{g. streptomycin/ml.}$  The unknown was adjusted to as near  $4 \mu\text{g./ml.}$  as possible, and three parts of both the standard and unknown solution were diluted each with one part of the diluting fluid. These four solutions were then compared in a  $4 \times 4$  Latin square, in which the two timing errors were incorporated in the rows and columns. Each plot in the square contained two tubes so that eight tubes were used for each solution. Each reading was squared and the averages of these figures were taken. Let the zones produced by the standard solutions be  $y_{11}$  and  $y_{12}$  and the zones produced by the unknown  $y_{21}$  and  $y_{22}$ . Let  $d$  be the difference between the  $\log_{10}$  concentrations of the two standard and unknown

solutions, i.e. 0.12494, and let  $M$  be the difference between the  $\log_{10}$  concentrations of standard and unknown. Then (Finney, 1944),

$$R = \frac{1}{2} (y_{22} - y_{21} + y_{12} - y_{11}), \quad T = \frac{1}{2} (y_{22} - y_{21} - y_{12} + y_{11}),$$

$$S = \frac{1}{2} (y_{22} + y_{21} - y_{12} - y_{11}), \quad M = -Sd/R.$$

$$\text{Standard error of } M = \pm \frac{sd}{R^2} \sqrt{\frac{R^2 + S^2}{n}}.$$

$T$  is a test of parallelism and its standard error is  $s/\sqrt{n}$ ;  $n$  = the number of tubes used for each concentration, i.e. 8;  $s^2$  = the error term derived from the analysis of variance of the squared figures. Since the range of the response is small this is a reasonable approximation.

Table 6. *Results of four accurate assays*

| Assay | Estimated streptomycin<br>concentration<br>( $\mu\text{g./ml.}$ ) | True streptomycin<br>concentration<br>( $\mu\text{g./ml.}$ ) | Actual<br>error<br>(%) | Estimated error<br>$2 \times \text{s.e. of mean}$<br>(%) |
|-------|---|--|------------------------|--|
| 1     | 3.445   | 3.466  | -0.6                   | $\pm 5$  |
| 2     | 4.264   | 4.231  | +0.8                   | $\pm 5$  |
| 3     | 3.744   | 3.730  | +0.4                   | $\pm 4$  |
| 4     | 4.121   | 4.106  | +0.4                   | $\pm 4$  |

Four assays on these lines using known quantities of streptomycin dissolved in  $\text{m}/15$  phosphate buffer (pH 7.8) were done. Sixteen tubes for each solution were used instead of eight. The results are given in Table 6 together with the estimated and actual errors. In each case the unknown and standard lines were parallel within the limits of experimental error. The estimated error was between  $\pm 4$  and 5%, and the actual error was well within these limits. More complex arrangements for performing a number of assays simultaneously are clearly possible and would slightly increase the accuracy of the procedure.

## DISCUSSION

Conventional pour-plate methods involve using numerous controls. Large volumes of agar must be melted and distributed accurately into uniform plates. All the fluids to be assayed must be accurately measured at each application. All procedures need careful timing. The results must be read to at least the nearest 0.25 mm., which is difficult on a plate and may entail a subjective error. All these features require such an expenditure of time and material as to make them unsuitable as a routine procedure. By the use of tubes, which are easily set up, require a small volume of agar and can be read with no subjective error, it is hoped that these difficulties have been overcome. Further, the volume of fluid required for an assay has been considerably decreased. Such a procedure provides a reasonably accurate, reliable and practicable routine method of assaying streptomycin and has been used over a period of a year for many hundreds of estimations on various fluids.

We have found it difficult to make any comparison of the accuracy of this method with other agar diffusion methods (Loo, McGuire, Savage, Skell,

Thornberry, Erlich & Sylvester, 1945; Kornegay, Forgacs & Henley, 1946; Challinor & King, 1947), since in these insufficient data are given. However, in the method described by Brownlee, Delves, Dorman, Green, Grenfell, Johnson & Smith (1948), where a *Bacillus subtilis* spore suspension is used, the fiducial limits ( $P=0.05$ ) calculated for an assay performed under very similar conditions to those described for our accurate assay procedure are  $\pm 4\%$ . Clearly the two methods are very similar in their accuracy. We would, however, claim that ours is a far simpler procedure, is more flexible and requires less apparatus.

The streptomycin used in these experiments was the hydrochloride, Lot No. 594, manufactured by Merck and Co., Rahway, New Jersey, U.S.A., and was supplied by the Streptomycin in Tuberculosis Trials Committee of the Medical Research Council. References to the weight of streptomycin used indicate the weight of the base.

## APPENDIX

### *The relation between depth of zone of inhibition and streptomycin concentration*

Results from the experiment detailed in Table 3 were used to investigate the relation between the depth of the zone of inhibition and the streptomycin content of the fluid being assayed. The average of the readings was taken for each streptomycin concentration. At the same time each of the readings was squared and the average of these taken for each streptomycin concentration. The averages for both the squared and the unsquared values have been plotted against  $\log_{10}$  streptomycin concentration in Fig. 4. Before analysing these results in detail certain theoretical aspects may be considered.

Most processes of linear diffusion can be described by Fick's well-known differential equations:

$$\frac{\partial C}{\partial T} = D \frac{\partial^2 c}{\partial x^2}, \quad (1)$$

where particles in a concentration of  $c$  diffuse over a distance  $x$  in time  $T$ .

In considering the diffusion of streptomycin under the experimental conditions described it is found that there is a series of different solutions of this equation according to the boundary conditions chosen. Some of these boundary conditions are known while others are doubtful. The conditions assumed are as follows:

(1) That a process of diffusion occurs in the test fluid as well as in the agar column. This is likely, since the diameter of the tube is small and the tube is incubated at a constant temperature.

(2) That the two columns can each be considered as of infinite length. Experimentally it has been shown that the length of the column of the test fluid (as long as it is greater than about 1 mm.) does not affect the depth of the zone of inhibition. In similar experiments the length of the agar column has also been shown to be immaterial.



(8) That the rate of diffusion is the same in the test fluid as it is in the agar column. This is not, in fact, true, but the rates are probably very similar since the agar is only a 1 % gel.

(4) That there is no diffusion potential due to the charge of the streptomycin ion. Lenher & Smith (1936) have shown that no potential gradient arises when

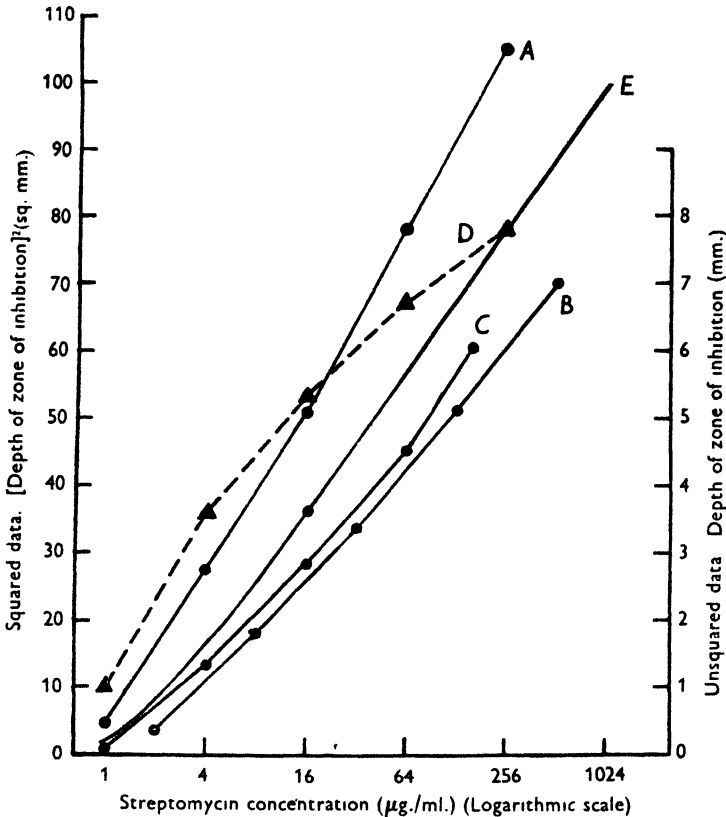


Fig. 4. Curves relating the zone of inhibition to the streptomycin concentration in the assay fluid. A, streptomycin made up in M/15 phosphate buffer pH 7.8. Squared data. B, streptomycin made up in pooled human serum. Squared data. C, streptomycin made up in M/15 phosphate buffer pH 7.8. Results of Latin square experiment detailed in Table 3. Squared data. D, the same results as in C but the data are not squared. E, calculated curve from equation (2) substituting  $C=0.35$  and  $2\sqrt{(DT)}=4.15$ .

charged dyes diffuse into water in the presence of a few equivalents of sodium chloride. We can therefore assume that the streptomycin ion behaves like an uncharged molecule.

(5) That the composition of the seeded agar is the same throughout its depth. The concentration at which streptomycin inhibits the growth of the test organism is, in part, determined by the composition of the medium in which growth occurs (May, Vourekha & Fleming, 1947). Constituents of serum or other body fluid, of which the streptomycin content is being determined, will diffuse into the agar column and establish a gradient. The composition of the

column must therefore vary throughout its length. However, if serum and buffer solution at pH 7.8 are used as test fluids, the curves relating the concentration of streptomycin that they contain to the depth of the zone of inhibition differ in height and slope but still have the same shape (Fig. 4). We have no means of estimating the effect of an osmotic gradient between the test fluid and the agar.

On the basis of these assumptions the relation between depth of zone of inhibition  $y$ , and concentration of streptomycin in the test fluid  $C_0$ , can be expressed (Mellor, 1912) by

$$C = \frac{C_0}{2} \left[ 1 - \Phi \left( \frac{y}{2\sqrt{(DT)}} \right) \right], \quad (2)$$

where

$$\Phi = \frac{2}{\sqrt{\pi}} \int_0^y e^{-v^2} dv,$$

$C$  = concentration of streptomycin necessary to inhibit the test organism,  
 $T$  = the time between adding the test fluid and the formation of the zone,  
 $D$  = the diffusion coefficient of streptomycin defined in (1). Cooper & Woodman (1946), in discussing the diffusion of antiseptics through agar gels, have used the expression

$$C = C_0 e^{-y^2/4DT}. \quad (3)$$

This expression assumes that the concentration of the antiseptic in the test fluid remains constant.

Vesterdal (1947), in considering radial diffusion of penicillin, used the expression

$$C = \frac{AC_0}{\sqrt{(\pi)DT}} e^{-y^2/4DT}, \quad (4)$$

where  $A$  is the cross-sectioned area of the Oxford cylinder. This expression assumes that the total quantity of the penicillin is initially deposited in an infinitely thin layer on the surface of the agar.

The fundamental resemblance between equation (2) and equations (3) and (4) can be brought out using the well-known series for  $\Phi(\theta)$  (Levy & Roth, 1936)

$$\Phi(\theta) = 1 - \frac{e^{-\theta^2}}{\theta\sqrt{\pi}} \left( 1 - \frac{1}{2\theta^2} + \frac{1.3}{(2\theta^2)^2} - \frac{1.3.5}{(2\theta^2)^3} + \dots \right). \quad (5)$$

If  $\theta$  is fairly large so that we may neglect terms of order  $\theta^{-2}$ , then

$$\Phi(\theta) \approx 1 - \frac{e^{-\theta^2}}{\theta\sqrt{\pi}},$$

so that, from (2), putting  $\theta = \frac{y}{2\sqrt{(DT)}}$ , we have, on rearranging,

$$\log C_0 = \log(2C\sqrt{\pi}) + \log \left( \frac{y}{2\sqrt{(DT)}} \right) + \frac{y^2}{4DT},$$

when  $y$  is large  $\log \left( \frac{y}{2\sqrt{(DT)}} \right)$  becomes negligible in comparison with  $\frac{y^2}{4DT}$ , and an approximately linear relation exists between  $\log C_0$  and  $y^2$ .

When  $y$  is small this resemblance breaks down; for we have (Levy & Roth, 1936)

$$\Phi(\theta) = \frac{2}{\sqrt{\pi}} \left( \theta - \frac{\theta^3}{1!3} + \frac{\theta^5}{2!5} - \dots \right), \quad (6)$$

whence, ignoring terms in  $y^2$ , using (2) and rearranging,

$$\log C_0 = \log (2C) + \frac{y}{\sqrt{(\pi DT)}},$$

so that  $\log C_0$  is linearly related to  $y$ , not to  $y^2$ . It is doubtful whether any of the proposed formulae hold for small values of  $y$ , and from the preceding discussion it seems dangerous to deduce values for  $D$  from the constants of fitted lines.

Since it is not possible to define the boundary conditions with certainty it is clearly unwise to say that these relationships hold accurately. We hope to show from experimental results that

(1) there is a close approximation to a linear relationship between  $y^2$  and  $\log C_0$ ,

(2) there is, however, a definite deviation from linearity at low concentrations of streptomycin.

These conclusions would indicate that equation (2) is a better solution than (3) or (4), since both of the latter equations stipulate a strictly linear relation between  $y^2$  and  $\log C_0$ . It would be difficult to fit experimental values to equation (2), but, by substituting values of 0.35 for  $C$  and 4.15 for  $2\sqrt{(DT)}$ , a curve can be drawn in the same range as two experimental curves (Fig. 4), and it is clear that it has a similar shape. We will now try to show that the results of the Latin square experiment of Table 3 yield these results.

The use of the squared data complicates the analysis somewhat, since the variance of the squared values is related closely to their mean at any given dose level, which invalidates the usual tests of significance. Under these circumstances a system of weighting must be used for deriving the error of the estimates. If one was using the unsquared data, the analysis of variance is as in Table 7. From this it is evident that the unsquared data depart significantly from linearity. The test for linearity using the squared figures has to be approached in a somewhat different manner, since the mean responses at each concentration level are of unequal accuracy and a weighted regression must be fitted. The method of doing this follows closely that worked out for probit analysis, of which several good accounts have been published (Finney, 1947*a*, *b*). If in the terminology of probit analysis we write 'response' for 'probit', then the following relationships hold:

- (1)  $Y$  = response (squared reading from provisional line),
- (2)  $Y_M = Y - 2Y$  (minimum working response),
- (3)  $R = 2\sqrt{Y}$  (range),
- (4)  $u$  = unsquared observed mean response,
- (5)  $Y_w = Y - 2Y + 2u\sqrt{Y}$  (working response) =  $Y - 2\sqrt{Y}(\sqrt{Y} - u)$ ,
- (6)  $W = \frac{1}{4}Y$  (weight).

The procedure is then to draw a line by eye to fit the squared responses (mean of the squared readings). From this the provisional values,  $Y$ , are taken and the working responses calculated. A weighted regression of  $Y_w$  on  $C_0$  is then fitted in the usual manner using weights  $nw$ , where  $n$  is the number of tubes at each concentration level. If necessary a second approximation can be

carried out calculating the  $Y'_w$  values from the  $Y'$  of the first line. The procedure is set out in Table 8.

The test of linearity now consists of comparing the sum of squares of the deviations of the observed point from the fitted line multiplied by  $n$  (the number of tubes at each concentration) with the error term of the analysis of variance. The line fitted to the squared data is based on the upper four con-

Table 7. *Analysis of variance of Latin square experiment* (Table 3)

|                             | Sum of<br>square | Degrees<br>of<br>freedom | Mean<br>square | <i>F</i> | <i>t</i> | <i>P</i>   |
|-----------------------------|------------------|--------------------------|----------------|----------|----------|------------|
| Between concentrations:     |                  |                          |                |          |          |            |
| Linear regression           | 280.2276         | 1                        | 280.2276       | —        | —        | —          |
| Quadratic regression        | 8.5511           | 1                        | 8.5511         | —        | 15.8     | <0.001     |
| Deviations from regressions | 0.5293           | 2                        | 0.2646         | 7.74     | —        | 0.01–0.001 |
| Rows                        | 0.2300           | 4                        | 0.0575         | 1.68     | —        | 0.2        |
| Columns                     | 0.4640           | 4                        | 0.1160         | 3.39     | —        | 0.2–0.05   |
| Interactions                | 0.6830           | 12                       | 0.0562         | 1.64     | —        | 0.2–0.05   |
| Error                       | 0.8550           | 25                       | 0.0342         | —        | —        | —          |

For the four higher concentrations:

|                             |         |   |         |   |      |           |
|-----------------------------|---------|---|---------|---|------|-----------|
| Linear regression           | 96.3272 | 1 | 96.3272 | — | —    | —         |
| Quadratic regression        | 0.2671  | 1 | 0.2671  | — | 2.73 | 0.02–0.01 |
| Deviations from regressions | 0.0008  | 1 | 0.0008  | — | —    | —         |

Table 8. *Analysis of squared data from Latin square experiment* (Table 3)

| 1      | 2    | 3    | 4       | 5         | $Y_u$  | $W$     | $WY_w$  |
|--------|------|------|---------|-----------|--------|---------|---------|
| $x$    | $u$  | $Y$  | $W=4Y$  | $2Y(Y-u)$ | (3.5)  | —       | —       |
| 1      | 3.63 | 14.5 | 0.01724 | +1.372    | 13.128 | 0.01724 | 0.22633 |
| 2      | 5.31 | 29.3 | 0.00853 | +1.082    | 28.418 | 0.01706 | 0.24076 |
| 3      | 6.71 | 44.3 | 0.00564 | –0.066    | 44.966 | 0.01692 | 0.25374 |
| 4      | 7.79 | 59.0 | 0.00424 | –1.690    | 60.690 | 0.01695 | 0.25714 |
| Totals |      |      | 0.03565 |           |        | 0.06817 | 0.97797 |

$$n=10 \quad \frac{1}{S(nW)} = 0.3565 \quad x=1.9125 \quad y=27.431$$

$$SW(x-x)^2 = 0.03955 \quad b = 15.8599$$

$$SWx(y-\bar{y}) = 0.62726 \quad y = y + b(x-x)$$

$$= 27.431 + 15.8599(x - 1.9125)$$

$$nS(u-\sqrt{Y})^2 = 0.053, \quad s^2 = 0.0342, \quad F = 1.55, \quad P = > 0.2.$$

Variance of estimated value at  $x=0$  is

$$V(Y_0) = s^2 \left\{ \frac{1}{S(nW)} + \frac{\bar{x}^2}{SnW(x-x)^2} \right\} = 0.0438.$$

Variance of difference

$$\bar{y}_0 - Y_0 = V(y_0) + V(Y_0) = 0.0780, \quad t = 10.4, \quad P = < 0.001.$$

centrations (256, 64, 16 and 4  $\mu\text{g./ml.}$ ), since it is impossible to include the point at 1  $\mu\text{g./ml.}$  in any reasonable provisional line without making the response negative. From Table 6 it will be seen that the observed value departs significantly from the line fitted to the remaining four points. It is therefore assumed that the relation between response and dose is not linear over the whole range. The proper comparison of the linearity of the squared and

and 
$$\text{Var } (\bar{y}_3) = \frac{4 \times 29.4425 \times 0.0222}{4}$$

$$= 0.6536.$$

So 
$$\text{Var } (\bar{y}) + \text{Var } (\bar{y}_3) = 0.1540 + 0.6536$$

$$= 0.8076.$$

now 
$$\text{Var } (b) = \frac{4 \times 0.0222 \times 64.0016}{8.6995}$$

$$= 0.6553 \quad \text{from } (12a),$$

so we can now calculate

$$\sigma_{x_3}^2 = \frac{0.8076}{31.8964} + \frac{(50.9033 - 13.0983)^2 \times 0.6533}{971,672}$$

$$= 0.000819 + 0.000961$$

$$= 0.00178,$$

and we have finally

$$x_3 = 1.1231 \pm 0.0422.$$

Using the value of Student's  $t$  for the 5 % point on 10 degrees of freedom we find the limits of error of  $M$  as

$$1.0291 - 1.2216,$$

giving an experimental error of about 8 % in logarithmic units and of +27 % and -19 % in units of concentration. These limits are, of course, calculated for one particular experiment and can only be considered as typical of those likely to be found in other similar experiments. In a routine assay the fluids composing the controls and unknown are different and, as previously indicated, an increased estimate of the error must therefore be made.

A further statistical point which deserves to be mentioned is the very high degree of grouping which the experimental method imposes on the data. The readings are made to the nearest 0.1 mm. That is to say, the grouping unit is 0.1 mm., which is of about the same order of magnitude as the error standard deviation of the technique. For this reason it would be as well to have at least 5 degrees of freedom for the estimate of the error mean square. Sheppard's corrections should not be used when making tests of significance on the data.

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## The Effect of Artificial Fertilizers and Dung on the Numbers of Amoebae in Rothamsted Soils

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**SUMMARY:** The total numbers (active + cystic) and the numbers of active amoebae in plots treated with complete minerals + ammonium sulphate and with farmyard manure were much higher than in the untreated plots. The complete minerals plot of Barnfield had a just significantly lower total count of amoebae than the farmyard manured plot, although no significant difference existed between the counts of active amoebae. The difference in the numbers of both the total and the active amoebae between the complete minerals and farmyard manure-treated plots on Broadbalk was not significant. No correlation was found between the percentage of organic carbon in the soils and the number of amoebae.

The occurrence of amoebae and flagellates in large numbers in a normal soil, both in the active and cystic condition, is well known from the work of Cutler, Crump & Sandon (1922) and others. Crump (1920) found that in one of the Rothamsted fields (Broadbalk) there were more Protozoa, especially amoebae, in the plot manured with farmyard manure than in the untreated one. It is generally believed that the presence of plenty of organic matter in soil will favour an extensive development of bacteria and fungi, which in their turn will be accompanied by an abundant development of Protozoa and other predators. To test the validity of this belief about amoebae, counts of these organisms were made on nine occasions from three of the plots from Barnfield and on six occasions from similarly treated plots of Broadbalk, Rothamsted. This work was started by Mr A. V. Garcia who made the first three counts given in Table 2.

### *Methods*

Twelve 4 in. borings were taken at intervals of about 2 yd. in a line across the plot and thoroughly mixed together to form a sample. The samples from the three plots were brought to the laboratory at the same time and passed through a 3 mm. sieve. The numbers of amoebae were estimated by the dilution technique of Singh (1946), eight replicate subsamples being tested from each dilution. Only cultures in which amoebae could be seen by microscopic examination were considered positive.

### *Statistical analysis of the data*

The analysis of the data in this paper is based on the theory developed by Fisher (cf. Introduction to Fisher & Yates (1947) and Appendix to Singh (1946)). The theoretical standard error of each count of negative cultures is approximately  $\sqrt{8}$ , and the distribution of the number of cultures may be taken as normal. Hence, ignoring the error due to soil sampling (see below), the 5% significance level difference between two individual counts is eight cultures,

corresponding to approximately 100% difference in population estimates. Since there was a different soil humidity on the different plots a correction was made for this. The estimates of population/g. were adjusted to a dry-weight basis before analysis. The first Barnfield observation contained no measurement of soil moisture and so was rejected.

*Count of amoebae from duplicate samples*

Recently it has been claimed by a few workers (see Harmsen, 1940; and references in James & Sutherland, 1942) that the method of soil sampling and the dilution technique used at Rothamsted and elsewhere for the plate count of bacteria in soil do not give reproducible results when several samples of a soil or different portions of a single soil sample are compared. This has led some to believe that the fluctuations in the numbers of bacteria in soil at short intervals reported from Rothamsted are due to faulty technique and do not really occur in soils in their natural state. It is not intended to discuss the matter here, but it may be pointed out that a few of the classical fields of Rothamsted which have been investigated have given reproducible results in the count of Protozoa and bacteria when several soil samples or fractions of a single sample have been compared by the techniques used at Rothamsted. This may be due to an unusual homogeneity of the soils from the Rothamsted classical fields as a result of similar cultivation and treatments carried out over a very long period. It would be of great interest to compare a variety of different soils to find out whether the elaborate methods of sampling and dilution technique suggested by a few workers are necessary in quantitative studies on soil micro-organisms. It is hoped to follow up this line of investigation.

To test the accuracy of sampling in the count of amoebae by the dilution technique several counts of the total amoebae in duplicate samples were made from different plots of Barnfield and Broadbalk (Table 1). Two samples, A and B, were taken at the same time on eight different dates and the number of amoebae determined. The differences between duplicate samples were not significant in any of the comparisons (cf. Singh (1946) for the standard error of such counts). The  $\chi^2$  test on these differences reveals no more variation than may be expected from the dilution method.

*Comparison of the numbers of amoebae in three different plots on Barnfield and on Broadbalk, Rothamsted*

The three plots selected from Barnfield (permanent mangolds) were: plot 8.0, untreated; plot 1.0, receiving 14 tons of dung/acre/year; plot 4 A, receiving a dressing of complete minerals (3½ cwt. of superphosphate, 500 lb. of potassium sulphate, 200 lb. of sodium chloride and 200 lb. of magnesium sulphate) and 412 lb. of ammonium sulphate/acre/year. These plots have been treated in the same way since 1876. Three similar plots were selected from Broadbalk (permanent wheat): plot 8, untreated; plot 2, receiving 14 tons of dung/acre/year; plot 7, receiving complete minerals (3½ cwt. of superphosphate, 2 cwt. potassium



sulphate, 1 cwt. sodium sulphate and 1 cwt. magnesium sulphate), and 4 cwt. of ammonium sulphate/acre/year. Plot 3 has been unmanured since 1889. Wheat has been grown continuously in each of the three plots since 1852, and the fertilizer treatments have been the same each year.

Table 1. *The total counts of amoebae (active + cystic) found when two samples were taken at the same time from Barnfield or Broadbalk plots*

F.Y.M. = farmyard-manured plot; C.M. = complete minerals + ammonium sulphate plot; U. = untreated plot;  $\Delta$  = difference in number of negative cultures between samples A and B.

| Date             | Samples | Total count of amoebae/g. wet soil |                    |                  |                | No. of negative cultures | $\Delta$ |
|------------------|---------|------------------------------------|--------------------|------------------|----------------|--------------------------|----------|
|                  |         | Barnfield (F.Y.M.)                 | Broadbalk (F.Y.M.) | Broadbalk (C.M.) | Broadbalk (U.) |                          |          |
| 4 August 1943    | A       | 26,700                             | —                  | —                | —              | 45                       | 5        |
|                  | B       | 17,800                             | —                  | —                | —              | 50                       |          |
| 6 August 1943    | A       | 41,400                             | —                  | —                | —              | 40                       | 3        |
|                  | B       | 31,800                             | —                  | —                | —              | 48                       |          |
| 20 January 1948  | A       | —                                  | 26,700             | —                | —              | 45                       | 8        |
|                  | B       | —                                  | 34,700             | —                | —              | 42                       |          |
| 10 February 1948 | A       | —                                  | —                  | 77,100           | —              | 33                       | 6        |
|                  | B       | —                                  | —                  | 45,200           | —              | 39                       |          |
| 2 March 1948     | A       | —                                  | —                  | —                | 18,800         | 49                       | 4        |
|                  | B       | —                                  | —                  | —                | 13,300         | 53                       |          |
| 2 April 1948     | A       | —                                  | 84,200             | —                | —              | 32                       | 3        |
|                  | B       | —                                  | 64,500             | —                | —              | 35                       |          |
| 27 April 1948    | A       | —                                  | —                  | 26,700           | —              | 45                       | 4        |
|                  | B       | —                                  | —                  | 37,900           | —              | 41                       |          |
| 23 June 1948     | A       | —                                  | —                  | —                | 11,000         | 55                       | 0        |
|                  | B       | —                                  | —                  | —                | 11,100         | 55                       |          |

Table 2 shows the results obtained from Barnfield plots. The total and the active counts of amoebae are very much lower in the untreated plot than in either the farmyard manured or the complete minerals + ammonium sulphate plots. Total counts (active + cystic amoebae) in the latter plots did not differ significantly from those in the farmyard-manured plot on any occasion. When the eight counts are taken together the farmyard-manured plot has about 30 % higher count of amoebae, the difference being just significant at the 5 % level. The differences between counts of active amoebae in the minerals and farmyard-manured plots were not significant, with the exception of one observation.

Table 3 shows the counts of amoebae taken on six occasions from the three corresponding plots of Broadbalk. Both the total and the active counts in the untreated plot are again very much lower than in the other two plots. The total counts in the farmyard-manured and complete-minerals plot did not differ significantly on five occasions, but on one occasion the farmyard plot had a significantly higher number. When all the counts are taken together, the farmyard plot has about 50 % higher total count, this difference not being statistically significant. Similarly, in the active counts there are no significant differences either on individual occasions or in the means of all the counts.

Table 2. *The numbers of amoebae/g. dry soil in untreated and treated plots of Barnfield at Rothamsted*

The first observation contains no measurement of soil moisture and has been omitted in calculating the means.

| Date            | Soil temperature<br>4 in. depth in °C. | Untreated plot (8-0) |        |        |                                  | Farmyard-manured plot (1-0) |        |         |                                  | Complete-minerals plot (4A) |        |         |                                  |
|-----------------|--|----------------------|--------|--------|----------------------------------|-----------------------------|--------|---------|----------------------------------|-----------------------------|--------|---------|----------------------------------|
|                 |  | Counts               |        |        | Soil H <sub>2</sub> O<br>content | Counts                      |        |         | Soil H <sub>2</sub> O<br>content | Counts                      |        |         | Soil H <sub>2</sub> O<br>content |
|                 |  | Total                | Cystic | Active |                                  | Total                       | Cystic | Active  |                                  | Total                       | Cystic | Active  |                                  |
| 24 March 1945   | 6.5                                    | 10,200               | 13,300 | —      | —                                | 26,700                      | 31,800 | —       | —                                | 17,300                      | 4,670  | 12,630  | —                                |
| 16 April 1945   | 13.7                                   | 5,530                | 1,270  | 4,260  | 15.5                             | 15,300                      | 8,300  | 7,000   | 20.5                             | 12,400                      | 1,300  | 11,100  | 17.5                             |
| 11 October 1945 | 11.4                                   | 14,000               | 2,070  | 11,930 | 13                               | 37,900                      | 2,550  | 35,350  | 16                               | 36,100                      | 1,440  | 34,660  | 12                               |
| 27 April 1946   | 9.6                                    | 2,320                | 1,790  | 580    | 15.5                             | 18,100                      | 2,060  | 16,040  | 20                               | 13,200                      | 980    | 12,220  | 16                               |
| 13 May 1946     | 10.6                                   | 8,880                | 4,840  | 4,040  | 11.5                             | 38,800                      | 9,590  | 29,210  | 18                               | 26,700                      | 6,050  | 20,650  | 16                               |
| 28 May 1946     | 11.5                                   | 18,000               | 4,100  | 13,900 | 19.5                             | 53,100                      | 3,550  | 49,550  | 22                               | 45,700                      | 6,670  | 39,030  | 17                               |
| 20 June 1946    | 11.8                                   | 8,480                | 1,940  | 6,540  | 15                               | 40,000                      | 5,870  | 34,130  | 20.5                             | 22,100                      | 3,550  | 18,550  | 15                               |
| 8 July 1946     | 17.4                                   | 8,060                | 4,020  | 4,040  | 10.5                             | 63,500                      | 11,000 | 52,500  | 15                               | 32,600                      | 7,370  | 25,230  | 10.5                             |
| 27 August 1946  | 15.0                                   | 12,500               | 3,730  | 8,770  | 11.5                             | 32,800                      | 10,500 | 22,300  | 18.5                             | 38,500                      | 5,660  | 32,840  | 17.5                             |
| Means           | 11.9                                   | 8,000*               | 2,700* | 5,300* | 14.0                             | 34,000*                     | 6,000* | 28,000* | 18.8                             | 26,000*                     | 3,000* | 23,000* | 15.2                             |

\* These means are derived from the mean number of negative cultures and are therefore not the arithmetic means of individual estimates.

Table 3. *The numbers of amoebae/g. dry soil in untreated and treated plots of Broadbalk at Rothamsted*

| Date             | Soil temperature<br>4 in. depth<br>in °C. | Untreated plot (3) |        |         |                                  | Farmyard-manured plot (2) |         |         |                                  | Complete-minerals plot (7) |        |         |                                  |
|------------------|---|--------------------|--------|---------|----------------------------------|---------------------------|---------|---------|----------------------------------|----------------------------|--------|---------|----------------------------------|
|                  |   | Counts             |        |         | Soil H <sub>2</sub> O<br>content | Counts                    |         |         | Soil H <sub>2</sub> O<br>content | Counts                     |        |         | Soil H <sub>2</sub> O<br>content |
|                  |   | Total              | Cystic | Active  |                                  | Total                     | Cystic  | Active  |                                  | Total                      | Cystic | Active  |                                  |
| 20 January 1948  | 0.7                                       | 16,400             | 5,280  | 11,120  | 18.99                            | 34,100                    | 12,250  | 21,850  | 23.45                            | 33,600                     | 4,160  | 29,440  | 20.59                            |
| 10 February 1948 | 5.1                                       | 13,200             | 6,030  | 7,170   | 15.72                            | 137,000                   | 11,710  | 125,290 | 19.88                            | 93,000                     | 17,500 | 75,500  | 17.08                            |
| 2 March 1948     | 3.3                                       | 21,600             | 4,510  | 17,090  | 13.16                            | 55,700                    | 19,500  | 36,200  | 18.84                            | 31,100                     | 5,920  | 25,180  | 14.24                            |
| 2 April 1948     | 5.1                                       | 20,400             | 5,500  | 14,900  | 15.12                            | 80,100                    | 27,800  | 52,300  | 19.48                            | 41,800                     | 8,680  | 33,120  | 16.92                            |
| 27 April 1948    | 11.3                                      | 22,400             | 8,580  | 13,820  | 8.36                             | 45,900                    | 17,500  | 28,400  | 9.76                             | 41,500                     | 14,700 | 26,800  | 8.08                             |
| 23 June 1948     | 13.3                                      | 13,000             | 13,000 | 0       | 14.40                            | 146,000                   | 45,900  | 100,100 | 17.36                            | 69,800                     | 6,010  | 63,790  | 15.43                            |
| Means            | 6.4                                       | 17,000*            | 7,000* | 10,000* | 14.29                            | 72,000*                   | 20,000* | 52,000* | 18.13                            | 48,000*                    | 8,000* | 40,000* | 15.50                            |

\* These means are derived from the mean number of negative cultures and are therefore not the arithmetic means of individual estimates.

In general the results on Broadbalk confirmed those from Barnfield in spite of a much higher population in the former. The following conclusions may thus be drawn with some confidence. Although the percentage of organic carbon is roughly the same in the untreated and complete-minerals plots of Barnfield and Broadbalk, the number of amoebae in the untreated plots is much lower than in the complete-minerals plots. The differences in the total counts as

Table 4. *The pH values and organic matter content of the soils in the different plots of Barnfield and Broadbalk at Rothamsted.*

2 mm. air-dry soil was used to determine the percentage of organic content of the soils

| Plot                        | pH value | %   |
|-----------------------------|----------|-----|
| <b>Barnfield</b>            |          |     |
| Untreated (plot 8.0)        | 7.2      | 0.8 |
| Complete minerals (plot 4A) | 7.1      | 0.8 |
| Farmyard manured (plot 1.0) | 7.0      | 2.5 |
| <b>Broadbalk</b>            |          |     |
| Untreated (plot 3)          | 8.1      | 1.1 |
| Complete minerals (plot 7)  | 7.9      | 1.2 |
| Farmyard manured (plot 2)   | 7.7      | 2.6 |

between the complete-minerals plots and the farmyard-manured ones are not statistically significant in the Broadbalk observations, and just reach significance in the case of Barnfield, in spite of large differences in the organic matter contents between these two soils (Table 4). The differences between counts of active amoebae are not significant.

It is of interest that the yields of mangolds and wheat in the untreated plots are about one-third those of the farmyard manure or the complete-minerals plots, as are also the numbers of amoebae in these plots. No marked differences are found in the yields of the plots treated with farmyard manure or with complete minerals + ammonium sulphate. Thus the crop yields and the population of amoebae are correlated in both fields. It may be emphasized that the treatment of the soil with artificial fertilizers for a long time has had no detrimental effect on the amoebae, but has in fact much increased their numbers in comparison with those in the untreated plot.

#### DISCUSSION

It has been claimed by some earlier workers that the numbers of Protozoa, especially amoebae, in soil is insufficient to cause changes in the bacterial population. Daily estimates made by Cutler *et al.* (1922) over a period of 365 days, however, indicated amoebae in numbers which, in view of their food requirements, must greatly affect bacterial numbers. Indeed, these authors obtained evidence of an inverse relationship between the numbers of active amoebae and those of bacteria as shown by plate counts. Improved technique (Singh, 1946) has since shown that the numbers of amoebae were previously underestimated. Soil amoebae and other groups of micropredators are selective in their bacterial food requirements on non-nutrient agar

(Singh, 1941, 1942, 1945, 1946, 1947 *a, b, c*, 1948). Differential feeding effects for certain strains of bacteria have also been shown to occur in sterilized soil both with true soil amoebae and with myxamoebae of Acrasieae (Singh, 1941, 1947 *c*). This work suggests that the micropredators may also affect the quality of the bacterial population, or conversely that their numbers may be affected by the proportion of edible bacteria present in soil.

The present work shows that the differences in the numbers of active amoebae as between the complete minerals and the farmyard-manured plots of Barnfield and Broadbalk are not statistically significant. Parallel estimates, however (unpublished), have shown that the bacterial populations of farmyard-manured plots are higher than those in the complete-minerals plots, at least as estimated by plate counts. Thus it seems that some other factor, such as the quality of the bacterial flora, must account for the different relative effect of manurial treatment on bacteria and amoebae respectively.

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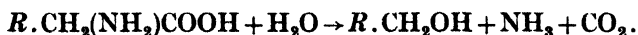
## The Assimilation of Glutamic Acid by Yeast

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**SUMMARY:** Using the corresponding amino-acid decarboxylases, the six amino-acids arginine, glutamic acid, histidine, lysine, ornithine and tyrosine were found to be free inside the cells of yeast. They are present when growth takes place in the absence of amino-acids, but their concentration may be increased by growing the organisms in media rich in amino-acids. Uptake of glutamic acid from the external medium is dependent on a source of energy which can be provided by the simultaneous fermentation of glucose. The presence of an ammonium salt in the medium decreases both the rate at which glutamic acid enters the cells and the amount of glutamic acid which can be taken up. Certain other amino-acids exert a similar sparing action on the assimilation of glutamic acid, which can be related to their efficiency as nitrogen sources for growth. The free glutamic acid content of cells remains practically constant when the cells are suspended in salt solutions without amino-acids, but if glucose is also present then the concentration of free glutamic acid inside the cells decreases steadily. When ammonia is also present the concentration of glutamic acid remains constant, suggesting that glutamic acid is synthesized by the cells under these conditions, or alternatively that the ammonia is assimilated and utilized preferentially.

Ehrlich (1907, 1909), in studies on the production of fusel-oil during yeast fermentation, showed that the organism used certain amino-acids as a source of nitrogen for growth. With a growth medium containing inorganic salts, sucrose and a single amino-acid as the sole source of nitrogen, he was able to isolate from the medium, when fermentation had ceased, either an acid or an alcohol corresponding to the amino-acid used. This result led him to suggest that yeast is able to split off carbon dioxide and ammonia from the amino-acid molecule, leaving an alcoholic residue which cannot be attacked further:



From Ehrlich's work it might appear that the sole function of amino-acids in yeast nutrition is to provide a source of ammonia, which in every case is the ultimate source of nitrogen for yeast growth regardless of the amino-acid used.

These experiments were confirmed more recently by Thorne (1937), who extended them to include several amino-acids not tested by Ehrlich. Thorne has shown that all amino-acids are not equally effective as sources of nitrogen for yeast growth. Some, such as aspartic and glutamic acids, are better than ammonium phosphate, others support slower growth, and some are attacked by yeast so slowly that they are useless as sources of nitrogen for growth. Several explanations of these findings were suggested. Some of the amino-acid degradation products are inhibitors of growth and fermentation, particularly the alcohol tryptophol, which is inhibitory in a concentration of  $10^{-3}$  M (Thorne, 1939). The production of inhibitory substances cannot be correlated with the effect on growth in all cases, however, and Thorne (1939) suggests that the

chief reason for these differences lies in the ease with which the amino-acids can be deaminated by the yeast to liberate ammonia. Since mixtures of amino-acids are more readily attacked than would be expected from results obtained with the separate components, Thorne (1945) suggests that yeast may bring about reactions between pairs of amino-acids in a manner analogous to that found by Stickland (1934) to occur with *Clostridium sporogenes*.

The amino-acid decarboxylases provide a means of estimating certain amino-acids both inside the cells of micro-organisms, in the medium, or on the surface of the cells (Gale, 1947). Gale studied the passage of amino-acids, particularly glutamic acid and lysine, across the membrane of the streptococcal cell and showed that mechanisms exist whereby these amino-acids are concentrated inside the cell. He points out that this mechanism would be of greater value to organisms that are nutritionally exacting and unable to synthesize their own amino-acid requirements than to organisms that are able to do so. Stokes & Gunness (1946) and Freeland & Gale (1947) estimated the amino-acid composition of certain micro-organisms, including yeasts, and showed that the amino-acid composition of the cell proteins, although differing for different organisms, was unaffected by very varied growth conditions. In a survey of the ability of a wide range of organisms to assimilate amino-acids it was shown (Taylor, 1947) that the yeasts and all Gram-positive bacteria tested contained significant amounts of free amino-acids, but that no free amino-acids could be detected in the cells of Gram-negative bacteria. In the case of yeast which grows with an ammonium salt as the sole nitrogen source, and which may attack amino-acids solely in order to obtain ammonia from them, it was felt that a study of the conditions under which free amino-acids are found inside these cells might throw some further light on the problem of nitrogen assimilation by such organisms.

#### EXPERIMENTAL

The two organisms used for the work described in this paper were obtained from the Carlsberg collection: Yeast foam (No. 237) and Dutch Top Yeast (No. 174).

##### *Growth of the yeasts*

**Medium 1.** Inorganic salt medium (Stephenson, 1936) at half the strength used for bacteria; tryptic digest of casein to give final concentration equivalent to 1.5 % casein; 0.2 % (w/v) Difco yeast extract; 4.0 % (w/v) glucose; pH adjusted to 5.5 before autoclaving.

**Medium 2.** As medium 1 but with the casein digest omitted.

**Medium 3.** As medium 1 but with both casein digest and Difco yeast extract omitted, and supplemented by the addition of trace elements and growth factors as follows.

(a) Growth factors (final concentration in  $\mu\text{g./ml.}$  medium), inositol, 1.0; calcium pantothenate, 0.2; nicotinic acid, 0.2; pyridoxin, 0.2; aneurin, 0.2; biotin, 0.001.

(b) Trace elements stock solution (g./l.):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 20.0; NaCl, 1.0;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$ , 0.5;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.05;

10 ml. 0.1 N-H<sub>2</sub>SO<sub>4</sub>. This solution was added in the proportion 10 ml./l. final medium.

The organisms were grown at 25° in Roux bottles each containing 150 ml. medium.

Dry weights of the organisms in cultures and washed suspensions were estimated turbidimetrically, using a photoelectric absorptiometer previously calibrated against suspensions of the organisms of known dry weight. In the case of cultures older than 48 hr., clumped growth rendered the turbidimetric method inaccurate, and the weights were obtained by drying samples to constant weight at 100°.

*Estimations.* Amino-acids were estimated manometrically using the specific decarboxylase preparations described by Gale (1945, 1946). These estimate the unsubstituted L-isomers only, and consequently the amino-acids used in the assimilation experiments were the natural L-isomers in all cases, although, as shown by Nielsen & Hartelius (1938), both D- and L-glutamic acid are completely assimilable by yeast.

Ammonia was estimated by a modification of the micro-diffusion method of Conway & Byrne (1933). The standard acid in the centre chamber of the Conway unit was replaced by approximately 0.1 N-HCl. When diffusion was complete, the acid was removed from the centre chamber, and transferred quantitatively with washings to a graduated cylinder, made up to a suitable volume and the ammonia estimated colorimetrically after the addition of Nessler's solution.

*General procedure.* The general technique used in studies on the variations in amino-acid content of the cells under various experimental conditions was as follows. The yeast, centrifuged down from the growth medium, was washed in M/15 potassium dihydrogen phosphate and made up into a thick suspension (20–100 mg. dry weight/ml.) in distilled water. The amino-acid content was assayed on a 1 ml. sample. The effect of various experimental procedures on the yeast was tested in buffered salt medium containing McIlvaine's citrate-phosphate buffer (final concentration 0.1 M) and Stephenson's (1936) inorganic salt medium (ammonia-free, the ammonium phosphate being replaced by disodium hydrogen phosphate) at the concentration used in the growth media. Yeast was added to give a tenfold dilution of the thick suspension, the diluted suspension was then shaken in a constant temperature bath, and the course of the reaction followed by removing portions of the suspension at intervals and analysing both supernatant medium and cells as described below.

## RESULTS

### *Effect of varying growth conditions*

Evidence that yeasts, in common with certain Gram-positive bacteria, contain significant amounts of free amino-acids, was obtained by a modification of Gale's (1947) technique; the amino-acid present in a cell suspension is assayed before and after rupture of the cell-wall. When assaying the amino-acids outside or on the surfaces of the intact cells, it was at first found necessary to



include manometric controls for the endogenous fermentation. It was discovered, however, that the external amino-acids are easily removed from yeast suspensions by washing, and it was therefore more convenient to eliminate fermentation controls by thorough washing of all yeast suspensions. The amount of amino-acid inside the cells can then be estimated after the cells have been ruptured by immersion of the suspension in boiling water for 20 min. Studies on the accumulation of amino-acids inside the cells of yeast were restricted to those six amino-acids which can be estimated by the decarboxylase technique.

Both yeasts (Table 1) contain all six amino-acids free in the cells, but the two organisms differ in the amounts of each amino-acid present, the most marked contrast being with lysine.

Table 1. *The free amino-acid content of yeast cells*

Cells grown for 24 hr. in medium 1.

|                 | Yeast Foam<br>( $\mu$ mol. amino-acid/100 mg. dry wt. cells) | Dutch Top Yeast |
|-----------------|--|-----------------|
| L-Arginine      | 4.24   | 5.80            |
| L-Glutamic acid | 10.36  | 17.10           |
| L-Histidine     | 1.43   | 2.59            |
| L-Lysine        | 3.89   | 42.00           |
| L-Ornithine     | 1.30   | 0.80            |
| L-Tyrosine      | 0.80   | 3.44            |

In order to determine whether the amounts of free amino-acids present varied during the course of growth, samples were taken at intervals from a large-scale culture grown in medium 1, and the results obtained with Dutch Top Yeast are shown in Fig. 1. The highest concentration of the four amino-acids tested is attained in the early stages of growth, and once growth becomes linear the content falls to a value which remains approximately constant even after growth has ceased.

In an attempt to discover whether the free amino-acids inside the cell vary in concentration according to the nature of the medium, cultures were grown in all three media. Medium 1 is rich, and medium 2 is deficient in free amino-acids, and medium 3 is free from amino-acids. Although the results (Table 2) show that the cell content is to some extent dependent on the external concentration, the yeast can still maintain a high concentration of certain amino-acids when grown in a medium where ammonia is the sole nitrogen source.

#### *Loss of amino-acids from the cells*

When yeast has been grown in medium 1 and has a high internal concentration of certain amino-acids, it seems likely that outward diffusion of those amino-acids might occur if the cells were resuspended in a medium free from amino-acids. To test this, a washed suspension of the yeast was assayed for arginine, glutamic acid and tyrosine and then added to 9 vol. of salt medium, as previously described. The dilute suspension was shaken in a constant temperature bath at 25°; portions were removed at intervals, the cells centri-

fuged down, and made up into a thick washed suspension again for assay. There was no significant loss of free amino-acids from the cells during 6 hr. When, however, glucose was added as a source of fermentable carbohydrate, the amino-acid content of the cells diminished (Table 3).

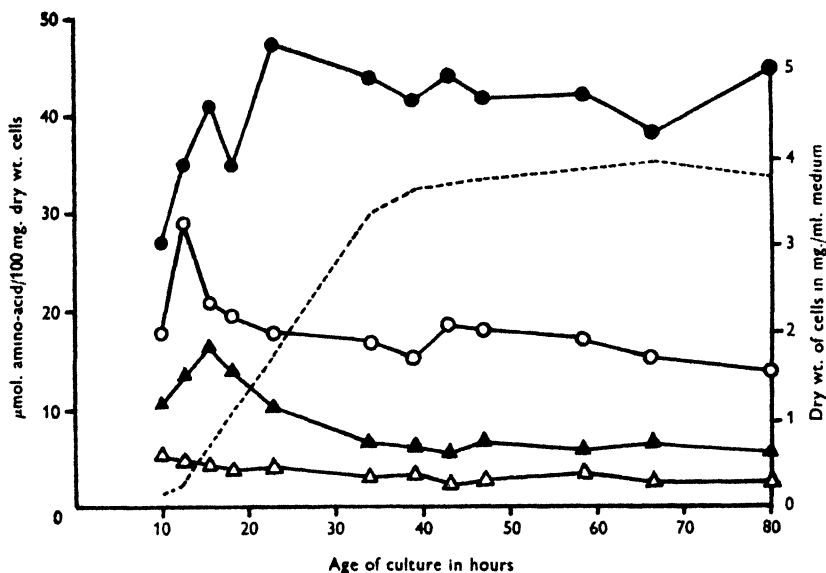


Fig. 1. Effect of age of culture on the free amino-acid content of Dutch Top Yeast. Dutch Top Yeast grown in medium 1 at 25°. Samples withdrawn at intervals from large-scale culture, growth estimated turbidimetrically and cells then centrifuged down. Amino-acid content of cells estimated and results expressed as  $\mu\text{mol.}$  of free amino-acid inside 100 mg. dry wt. of cells. -----, growth of yeast; -●-●-●-, lysine; -○-○-○-, glutamic acid; -▲-▲-▲-, arginine; -△-△-△-, tyrosine.

Table 2. Variation in free amino-acid content of Dutch Top Yeast with the nature of the growth medium

| Amino-acid      | Age of culture 24 hr.   |          |          |
|-----------------|---|----------|----------|
|                 | Growth on   |          |          |
|                 | Medium 1<br>( $\mu\text{mol. amino-acid/100 mg. dry wt. cells}$ ) | Medium 2 | Medium 3 |
| L-Arginine      | 11.80   | 2.37     | 1.38     |
| L-Glutamic acid | 18.00   | 8.35     | 8.57     |
| L-Histidine     | 2.77  | 2.19     | 0.58     |
| L-Lysine        | 46.80   | 2.86     | 0.89     |
| L-Ornithine     | 7.14  | 0.54     | 0.63     |
| L-Tyrosine      | 4.02  | 8.57     | 1.84     |

The disappearance of free amino-acids from the cells might occur in a number of ways. It is possible that (i) the amino-acids are able to diffuse out of the cells when fermentation is taking place simultaneously; (ii) the amino-acids are broken down inside the cell to provide ammonia for protein synthesis; (iii) amino-acids are metabolized inside the cell in other ways, such as transamination and direct synthesis into peptides and proteins. These possibilities were investigated in the case of glutamic acid in the following way.

The experiment described above was repeated under exactly similar conditions and glutamic acid estimated at each stage, both before (free amino-acid) and after (total amino-acid) acid hydrolysis of the sample. Although the free glutamic acid again decreased, the values obtained for the total (free + combined) glutamic acid showed that no change had occurred in the amount of combined glutamic acid during the whole course of the experiment. Disappearance of glutamic acid cannot therefore be accounted for by its being built up into peptide or protein.

Table 3. *Loss of amino-acids from the cells of Dutch Top Yeast*

Yeast grown for 24 hr. in medium 1 and crop incubated at 25° in buffered salt mixture at pH 5.0 (a) with and (b) without glucose. Yeast suspension 6.0 mg. dry wt./ml.

| Time<br>(hr.) | Amino-acid content of cells                 |                  |          |                 |                  |          |
|---------------|---|------------------|----------|-----------------|------------------|----------|
|               | Glucose absent                              |                  |          | Glucose present |                  |          |
|               | Arginine                                    | Glutamic<br>acid | Tyrosine | Arginine        | Glutamic<br>acid | Tyrosine |
|               | (μmol. amino-acid/100 mg. dry wt. of cells) |                  |          |                 |                  |          |
| 0             | 12.25                                       | 18.75            | 3.62     | 5.80            | 17.15            | 3.44     |
| $\frac{1}{2}$ | —   | —                | —        | 3.97            | 14.88            | 2.82     |
| 1             | 11.61                                       | 18.15            | 3.89     | 3.26            | 8.63             | 1.03     |
| 2             | 11.52                                       | 18.30            | 3.26     | 1.88            | 3.44             | 0.76     |
| 3             | —   | —                | —        | 0.98            | 1.68             | 0.31     |
| 4             | 11.80                                       | 18.40            | 3.53     | —               | —                | —        |
| 6             | 11.92                                       | 19.15            | 3.13     | —               | —                | —        |

To test the possibility of outward diffusion of the amino-acid, the test sample was centrifuged, and the supernatant and washings concentrated by vacuum distillation and assayed for glutamic acid. The results obtained were again negative, showing that loss of glutamic acid could not be accounted for by outward diffusion. The possibility that glutamic acid is deaminated to provide ammonia was tested by estimating succinic acid, the degradation product found by Erhlich (1909) and Thorne (1937), but the amount of succinic acid formed was independent of the amount of glutamate disappearing, and could be fully accounted for as being produced from glucose. No free ammonia could be detected at any stage.

The disappearance of glutamic acid from the yeast cell in the presence of glucose therefore appears to be the result of some internal metabolism, the nature of which is as yet obscure, but which probably consists of conversion into other amino-acids. Failure to detect succinic acid and free ammonia does not necessarily rule out the possibility of deamination, since breakdown may proceed only as far as  $\alpha$ -ketoglutaric acid and ammonia, both of which would enter into other reactions in the cell.

#### *Conditions affecting glutamic acid assimilation*

*Presence of glucose.* To investigate the conditions under which glutamic acid is assimilated by yeast, a washed suspension from a culture grown in medium 1 was assayed for internal glutamic acid and then incubated under the usual

conditions in the presence of 0.01 M glutamic acid both with and without the addition of glucose. In the presence of glucose the level of glutamic acid inside the cells increased steadily, but only a comparatively small increase occurred when glucose was omitted (Table 4). This small increase may have been brought about by glucose carried over from the growth medium or by the utilization of carbohydrate reserves by the cells; it was eliminated by subjecting the yeast suspension in salt medium to vigorous aeration for 4 hr. before incubation with glutamic acid.

Table 4. *Effect of the presence of glucose on the uptake of glutamic acid by yeast*

Cells (24 hr. culture) incubated in buffered salt medium with or without glucose (2% (w/v)). Yeast suspension 9.0 mg. dry wt./ml. Samples withdrawn at times shown, cells centrifuged down, washed, and internal concentration of free glutamic acid estimated. Results expressed as the change in free glutamic acid content—i.e. disappearance of free glutamic acid from the cells indicated by negative sign.

| Preparation   | Time<br>(hr.) | Change in free glutamic acid content                        |         |
|---|---------------|---|---------|
|   |               | Glucose   |         |
|   |               | Absent<br>( $\mu$ mol. amino-acid/100 mg.<br>dry wt. cells) | Present |
| (a) Cells centrifuged from<br>growth medium and<br>washed once in M/15 $\text{KH}_2\text{PO}_4$     | 0             | 0   | 0       |
|   | $\frac{1}{2}$ | 4.91  | 11.20   |
|   | 1             | 4.47  | 23.20   |
|   | 2             | 3.84  | 22.80   |
| (b) Cells treated as in (a)<br>then suspended in salt<br>medium and vigorously<br>aerated for 4 hr. | 0             | 0   | 0       |
|   | $\frac{1}{2}$ | -0.89   | 8.03    |
|   | 1             | 0.45  | 20.50   |
|   | 2             | -0.94   | 22.10   |

*pH of medium.* The pH of the suspending medium has no marked effect on the rate at which external supplies of glutamic acid can be assimilated. The pH curve for glucose fermentation by this organism is flat over the range pH 5.5–6.0, and the rate at which glutamic acid is assimilated is optimal at pH 4.5.

*Age of culture.* Since the amount of free glutamic acid inside the cells varies with the age of culture, it seemed likely that cultures of different ages would assimilate glutamic acid at different rates. Table 5 shows the results obtained when the capacity of a large-scale culture to assimilate glutamic acid was tested under standard conditions after 12, 24 and 48 hr. growth. Cells from a young culture take up glutamic acid more rapidly from the medium, and concentrate it internally to a higher degree at equilibrium than old cells do.

*External concentration of glutamic acid.* The effect of varying external concentrations of glutamic acid on the level attained inside the cell is shown in Fig. 2. In the presence of 0.01 M glutamic acid, the concentration inside the cell reached a steady value after incubation for 1 hr. At lower external concentrations, the rate at which glutamic acid is metabolized inside the cell is presumably greater than the rate at which it can be assimilated from the medium, and the internal concentration diminishes.

**Presence of an ammonium salt.** When yeast cells are incubated in glutamic acid solution, the amount which disappears from the external medium is equal

Table 5. *Variation with age of culture in the ability of yeast cells to assimilate glutamic acid*

Cells from cultures of different ages were incubated in salts-buffer-glucose mixture containing  $8.94 \mu\text{mol./ml.}$  glutamic acid. Yeast suspension  $10.5 \text{ mg. dry wt./ml.}$  Samples were removed at times shown, the cells centrifuged down and the amino-acid content of the supernatant estimated.

| Duration of exposure of cells to medium (min.) | Age of culture supplying cells (hr.)                             |      |       |
|--|--|------|-------|
|  | 12   | 24   | 48    |
|  | $\mu\text{mol. glutamic acid taken up/100 mg. dry wt. of cells}$ |      |       |
| 0  | 0  | 0    | 0     |
| 15   | 18.85  | 11.9 | 2.23  |
| 30   | 32.8   | 26.1 | 11.92 |
| 60   | 40.5   | 36.0 | 31.8  |
| 120  | 42.5   | 36.8 | 32.2  |

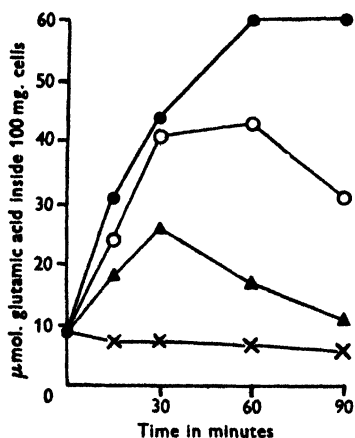


Fig. 2

Fig. 2. Effect of varying external concentrations of glutamic acid upon the glutamic acid content of yeast cells. Dutch Top Yeast grown for 24 hr. in medium 1. Cells incubated in salts-buffer-glucose mixture in the presence of varying external concentrations of glutamic acid. Temperature  $25^\circ$ . Yeast dry weight  $6.0 \text{ mg./ml.}$  External medium:  $\bullet\text{---}\bullet\text{---}\bullet$ ,  $8.98 \mu\text{mol. glutamic acid/ml.}$ ;  $\circ\text{---}\circ\text{---}\circ$ ,  $4.46 \mu\text{mol. glutamic acid/ml.}$ ;  $\blacktriangle\text{---}\blacktriangle\text{---}\blacktriangle$ ,  $2.23 \mu\text{mol. glutamic acid/ml.}$ ;  $\times\text{---}\times\text{---}\times$ , no external glutamic acid. Results expressed as  $\mu\text{mol.}$  free glutamic acid inside  $100 \text{ mg. dry wt. of cells.}$

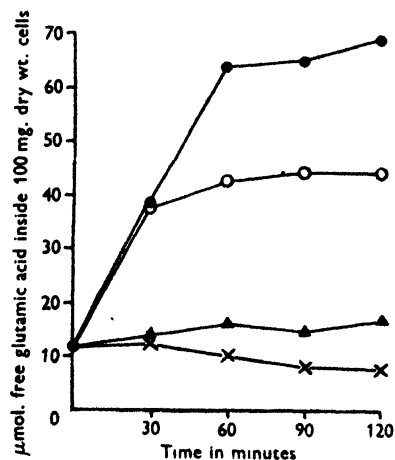


Fig. 3

Fig. 3. Effect of the presence of ammonia on the internal metabolism of glutamic acid and on the rate of glutamic acid uptake from the external medium. Dutch Top Yeast grown for 24 hr. at  $25^\circ$  in medium 1. Washed suspension of cells incubated in buffered salt medium at pH 4.5 containing 8% (w/v) glucose and  $3.7 \text{ mg. dry wt. yeast/ml.}$  Ammonia concentration  $14.28 \mu\text{mol. NH}_3\text{-N/ml.}$  Glutamic acid concentration  $8.98 \mu\text{mol./ml.}$  Results are expressed as  $\mu\text{mol. glutamic acid inside the cells of } 100 \text{ mg. yeast.}$   $\times\text{---}\times\text{---}\times$ , external medium salts-buffer-glucose only;  $\blacktriangle\text{---}\blacktriangle\text{---}\blacktriangle$ , + ammonia;  $\bullet\text{---}\bullet\text{---}\bullet$ , + glutamic acid;  $\circ\text{---}\circ\text{---}\circ$ , + ammonia + glutamic acid.

to that which appears inside the cells (Fig. 4a). When ammonia is added to the suspending medium as ammonium sulphate, the rate at which glutamic

acid is assimilated from the external medium is decreased, and a correspondingly diminished concentration of glutamic acid is found inside the cells (Fig. 4*b*).

In considering the importance of glutamic acid assimilation to the yeast cell and the effect which the presence of external supplies of ammonia has on this

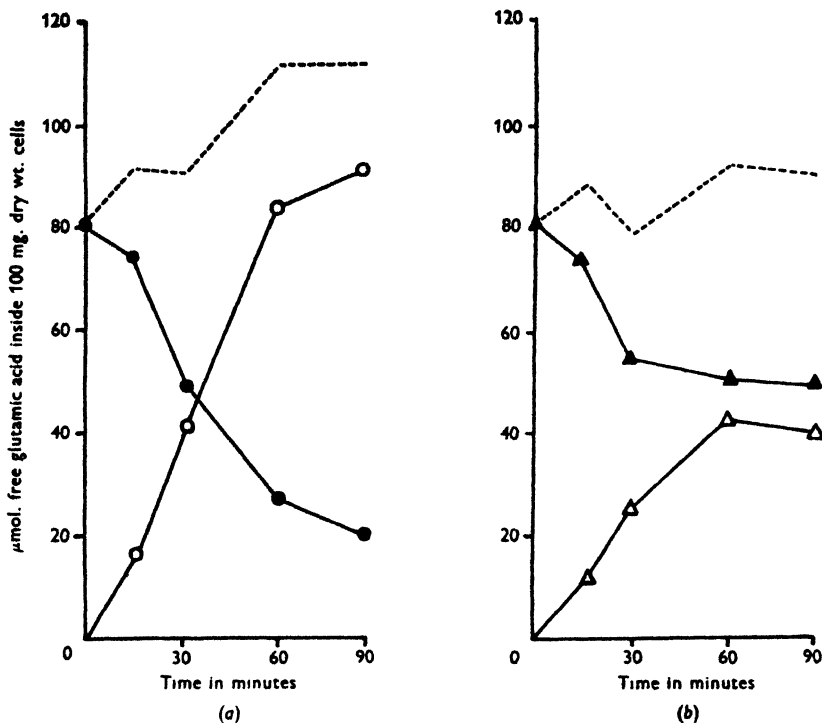


Fig. 4. Balance between glutamic acid inside the cells of Dutch Top Yeast and in the external medium (a) in ammonia-free salt medium, (b) in the presence of ammonia. Dutch Top Yeast grown for 24 hr. at 25° in medium 1. Washed suspension of cells dry wt. 5.6 mg./ml. incubated in buffered salt medium containing 2% (w/v) glucose under the following conditions: (a) (1) no additions, (2) + 8.92  $\mu\text{mol.}$  external glutamic acid/ml.; (b) (3) + 14.28  $\mu\text{mol.}$   $\text{NH}_3\text{-N/ml.}$ , (4) + 8.92  $\mu\text{mol.}$  glutamic acid and 14.28  $\mu\text{mol.}$   $\text{NH}_3\text{-N/ml.}$  Samples withdrawn at intervals, yeast centrifuged down, washed, and the glutamic acid content of the cells and of the supernatant medium in (2) and (4) estimated.  $\circ-\circ-\circ-$  represents the glutamic acid content of the cells in the presence of external supplies of glutamic acid, estimated by the difference between free glutamic acid content of the cells in (1) and (2) and again between (3) and (4) when ammonia is also present in the external medium. Results are expressed as  $\mu\text{mol.}$  glutamic acid inside 100 mg. dry wt. of cells.  $\bullet-\bullet-\bullet-$  represents the amount of glutamic acid disappearing from the external medium measured in (2) and (4), expressed as  $\mu\text{mol.}$  glutamic acid taken up by 100 mg. dry wt. of cells.  $-----$  represents the total free amino-acid present, glutamic acid disappearing from external medium + increase in free glutamic acid content of the cells.

process, it will be remembered that when growth takes place in a medium devoid of amino-acids, free amino-acids are still to be found inside the cells; the yeast must be able to synthesize all its amino-acid requirements from the ammonium salt. Fig. 3 shows that synthesis of glutamic acid takes place under these conditions. The concentration of glutamic acid diminishes when the cells

are incubated in the presence of glucose only, but if an ammonium salt is also present the concentration is maintained, and increases slightly. When the cells are incubated with glutamic acid, ammonia decreases the concentration of free glutamic acid inside the cells. It has been shown that the cells can synthesize glutamic acid from ammonium salts in the presence of glucose, and

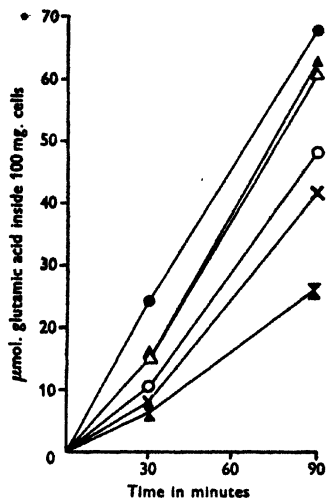


Fig. 5

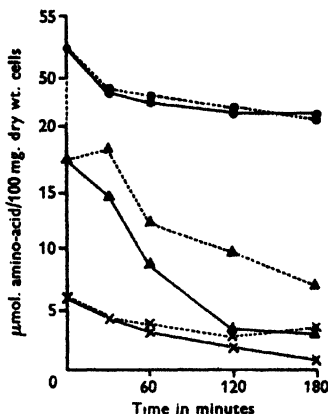


Fig. 6

Fig. 5. Effect of ammonia and certain amino-acids in decreasing the rate of glutamic acid assimilation by yeast. Dutch Top Yeast grown for 24 hr. at 25° in medium 1. Washed suspension of cells incubated in buffered salt medium at pH 4.5 containing 8.0% (w/v) glucose and 8.98 μmol. glutamic acid and 8.2 mg. dry wt. yeast/ml., with further additions: -●-●-●-, no additions; -○-○-○-, +14.28 μmol. NH<sub>3</sub>-N/ml.; -x-x-x-x-, +8.98 μmol. arginine/ml.; -Y-Y-Y-Y-, +8.98 μmol. aspartic acid/ml.; -▲-▲-▲-, +8.98 μmol. lysine/ml.; -Δ-Δ-Δ-, +8.98 μmol. tyrosine/ml. Results expressed as μmol. glutamic acid appearing inside 100 mg. dry wt. of cells.

Fig. 6. Loss of amino-acids from the cells of Dutch Top Yeast in the presence of glucose and ammonia. Dutch Top Yeast grown for 24 hr. at 25° in medium 1. Washed suspension of cells incubated at 25° in buffered salt medium at pH 4.5 containing 4% (w/v) glucose and 6.0 mg. dry wt. yeast/ml. Ammonia concentration 7.14 μmol. NH<sub>3</sub>-N/ml. Samples withdrawn at intervals and the free amino-acid content of the cells estimated. —, ammonia absent from external medium; ----, +ammonia in external medium; -●-●-●-, lysine content/100 mg. dry wt. cells; -▲-▲-▲-, glutamic acid content/100 mg. dry wt. cells; -x-x-x-x-, arginine content/100 mg. dry wt. cells.

it might be that the decreased assimilation in the presence of ammonia is the resultant of some sort of balance between assimilatory and synthetic processes; but other evidence, shortly to be published, rules out this possibility.

The effect in decreasing the rate of glutamic acid assimilation is not specific to ammonia. Fig. 5 shows that, whereas lysine and tyrosine have little effect on the uptake of glutamic acid, both arginine and aspartic acid have a greater sparing action on glutamic acid assimilation than ammonia itself. When these results are compared with the growth experiments of Thorne (1945) a definite

relationship is found. Aspartic acid is a better source of nitrogen for growth than glutamic acid or ammonia, and aspartic acid exerts a greater sparing action on the assimilation of glutamic acid than does ammonia itself. Lysine, on the other hand, which is virtually unattacked by yeast, has no significant sparing action.

Further evidence in support of this view was obtained by studying the concentrations of various amino-acids inside cells incubated with glucose both in the presence and absence of ammonia, as shown in Fig. 6. The high initial concentration of internal lysine does not decrease significantly over the incubation period, even in the absence of ammonia, and lysine consequently cannot be undergoing any metabolism; whereas the concentrations of both arginine and glutamic acid decrease as these compounds are metabolized, and ammonia exerts a definite sparing action in both cases.

### DISCUSSION

The present work shows that yeast contains the six amino-acids arginine, glutamic acid, histidine, lysine, ornithine and tyrosine in the free state inside the cell. This is so even when growth takes place in a medium free from amino-acids. When the sole source of nitrogen is an ammonium salt, synthesis of amino-acids must therefore take place inside the cell. By growing the organisms in a medium rich in free amino-acids, however, the concentration of amino-acids within the cell is increased, and from the effect of external glutamic acid on its concentration within the cell, it is clear that under conditions which exist in a medium containing amino-acids, this amino-acid is taken up from the external environment. Such assimilation will account for the higher values found for the amino-acid content of the yeast grown in a medium rich in amino-acid. For the assimilation, a source of energy, such as is provided by the simultaneous metabolism of glucose, is essential; and similarly glucose is necessary for the utilization of glutamic acid within the cell.

When cells rich in free amino-acids are suspended in salt media not containing amino-acids, there is no decrease in the internal concentration of glutamic acid until a source of energy is provided, when it begins to decrease steadily. This decrease could be accounted for in several ways, either by outward diffusion of the amino-acid or by its condensation into combined glutamic acid in peptide or protein, but the evidence rules out these possibilities. The glutamic acid which disappears may therefore be metabolized in some other way, and its utilization by the growing cell as the sole nitrogen source indicates that it can be converted into other amino-acids and nitrogenous compounds. It may be deaminated as the first step in such intracellular reactions and such a hypothesis is supported by the relationship between the effect of ammonia and other amino-acids in decreasing the rate at which glutamic acid is taken up from the external medium. Thorne (1939) suggested that the chief differences between the various amino-acids as effective sources of nitrogen for growth may be largely due to the ease with which yeast is able



to deaminate them; the present data on the effect of ammonia and various amino-acids on glutamic acid assimilation are not inconsistent with this suggestion.

The concentration of free amino-acids found inside the cell represents a balance between the rate at which they are utilized and the rate at which they are synthesized or taken up from the external medium (Gale & Mitchell, 1947). Consequently the appearance of free amino-acids inside a cell must mean that the amino-acids are either synthesized or taken from the medium at a rate greater than that at which they can be metabolized within the cell. This appears to be the case with glutamic acid in the yeast cell even when growing in medium free from amino-acids. The fact that the presence of ammonia decreases the concentration of free glutamic acid within the cell indicates that ammonia may (a) decrease the rate of glutamic acid uptake, (b) increase the rate of its internal metabolism, or (c) exert both effects. Possibility (b) is ruled out by the facts presented in Fig. 4. If ammonia and ammonia precursors act by 'sparing' glutamic acid, as shown by Fig. 4, then the primary action of ammonia must be on assimilation.

I wish to record my indebtedness to the late Dr Marjory Stephenson, F.R.S. and to thank Dr E. F. Gale for the help and encouragement they gave me throughout the course of this work. I am also indebted to the Medical Research Council for a personal grant.

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## Adaptation of *Sporocytophaga myxococcoides* to Sugars

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**SUMMARY:** Strains of *Sporocytophaga myxococcoides* were cultivated in a mineral nutrient medium with glucose. In media containing 0.1 % glucose sterilized by Seitz-filtration growth readily occurred, whereas in corresponding media with 0.5 and 1.0 % glucose there was a pronounced lag in development that sometimes lasted for several weeks.

The duration of the lag period appeared to depend on the number of cells in the inoculum, and could be diminished by incubation at 25° instead of 30°, although the latter temperature is nearer to the optimum temperature for growth. Apparently adaptation to the high glucose concentration takes place during the lag period.

Cells adapted to high glucose concentrations grow readily in media with the same concentration of mannose, whereas non-adapted cultures do not grow in these media, or only after a long lag period. None of the strains investigated showed any development in media in which either fructose or xylose was the sole source of carbon; nevertheless, these sugars did not inhibit, or only slightly, inhibited cellulose decomposition.

Stanier's observation, that the development of *Sp. myxococcoides* is inhibited when the glucose is autoclaved in the nutrient medium, was confirmed. If, however, due care be taken to avoid an alkaline reaction, glucose autoclaved either separately or in the medium sustains growth in the same way as does glucose sterilized by filtration.

For several types of cellulose-decomposing micro-organisms the ability to hydrolyse cellulose to the constituent sugars, either cellobiose or glucose, has been demonstrated. This, as well as the fact that at least some of these organisms have been found to grow on glucose as a sole source of carbon, has led to the general conception that cellulose breakdown by micro-organisms proceeds in the following two steps: (1) hydrolysis to glucose, succeeded by (2) oxidation or fermentation of this sugar to the final metabolic products. However, until recently cellulose-decomposing soil bacteria of the *Cytophaga* group seemed to be an exception to this rule. In spite of several attempts nobody had been successful in demonstrating either cellulase activity or growth on glucose. Moreover, the fact that even low concentrations (0.1–0.2 %) of reducing sugars, glucose included, retarded or even completely inhibited cellulose decomposition, suggested a toxic action of this sugar towards *Cytophaga* spp. As the older literature on this subject is discussed extensively in the papers of Stanier (1942*b*) and Fähræus (1947) we shall not enter into details here.

Fähræus (1941) attacked this problem from the quantitative side. With the aid of glucose and cellulose determinations he showed that glucose, added in low concentrations (0.05 %) to the cellulose medium, was rapidly consumed by both strains studied, and further that the observed delay in the cellulose decomposition was due to a preferential attack on the glucose. However, when using higher glucose concentrations he observed a lag in the development during which neither cellulose nor glucose was consumed. For the strain of

'*Cyt. globulosa*' received from Stapp, this occurred even in 0.1 % glucose, but Fåhræus's own strain '3' was less sensitive. After the initial growth inhibition, which lasted for about 10 days, glucose was consumed first, and then cellulose.

The demonstration that *Cytophaga* spp. were able to use glucose made it plausible that like other cellulose-decomposing organisms, they would hydrolyse this polysaccharide previous to oxidation. Further evidence for a hydrolytic breakdown was given later (Fåhræus, 1944, 1946, 1947).

Though the retardation of cellulose decomposition effected by low glucose concentrations had been satisfactorily explained by the fact that glucose is consumed preferentially, the phenomenon of the delay of both glucose and cellulose consumption caused by higher glucose concentrations required further investigation.

In 1942 Stanier published two papers on the *Cytophaga* group, dealing also with its glucose metabolism. At that time, owing to war conditions, Stanier was not yet acquainted with Fåhræus's results. The important discovery was made by Stanier that glucose not sterilized by heating, but by Seitz-filtration, was, at least in a liquid medium, an excellent source of carbon for his *Sporocytophaga* and *Cytophaga* strains. This appeared to be true for glucose concentrations up to 2 %, maximal growth occurring after 5 days in all concentrations, in tubes without cellulose. Stanier states in this connexion: 'Furthermore, the glucose sterilized by filtration exerted no "toxic effect" on the growth in the presence of cellulose. With the highest concentrations tested there was, it is true, a decrease in cellulose decomposition, but at the same time abundant growth occurred in the surrounding liquid medium. This partial inhibition of cellulose decomposition is probably due to preferential utilization of the monosaccharide.' One should compare these results with Stanier's experiments with glucose, autoclaved in the nutrient medium, where 0.1 % or less of glucose in the cellulose medium supported maximal growth after 6 days, but 0.5–2 % even after 20 days did not support any growth. Stanier suggested that the toxic effect must be a result of the formation of harmful decomposition products from the glucose when heated in the phosphate-containing medium. However, he did not describe experiments with glucose autoclaved separately in distilled water.

Fåhræus (1947) made it clear that throughout his earlier work he had used glucose sterilized in this way, and that he had nevertheless observed inhibition. From this, one might conclude that heating glucose in distilled water also gives rise to inhibiting substances. However, this need not necessarily be true, for in Fåhræus's experiments the glucose was toxic irrespective of any heating, since, contrary to Stanier, he observed inhibition also with Seitz-filtered glucose (cf. Fåhræus 1947, p. 58). The same results were obtained with different samples of glucose, purified glucose, and glucose prepared by hydrolysis of lichenin by means of enzyme preparations of *Cytophaga* spp. These experiments were all performed with Stapp's strain of '*Cyt. globulosa*'. Thus there remained a marked contrast between the results of Stanier and Fåhræus, which still had to be explained.

### Description of strains

Five strains were available. As all formed spherical microcysts and resembled *Sporocytophaga myxococcoides* Hutchinson & Clayton emend. Krzemieniewska (Stanier, 1940), in most other features, they may all be considered to belong to this species.

Two of the strains had been used by Fåhræus throughout his earlier experiments (1941, 1944, 1946, 1947), i.e. *Cytophaga* '3' and '*Cytophaga globulosa*'. The latter strain was isolated and described by Stapp & Bortels (1984).

The three other strains had been isolated in 1942 from garden soil by A. Kaars Sijpesteijn. These strains are designated H, W and D (Pl. 1, figs. 1-3). After preliminary purification in liquid media they were isolated in the following different ways. Strain H was obtained in pure culture by application of the dilution method, strain W by the cellulose agar technique, and strain D by 'pasteurization' for 10 min. at 70°. Microscopic examination of the cultures indicated the absence of any contaminants, and streaking on ordinary nutrient media did not give any growth, so that the purity of the cultures seemed guaranteed.

The length of the rods of these three strains is approximately the same, about 3-5.5  $\mu$ , and the diameter of their microcysts is approximately 1.6  $\mu$ . Strain D produces a pale yellow pigmentation and does not produce much slime, whereas strains H and W cause a deep yellow orange coloration of the paper accompanied with more abundant slime production.

The first author (A. K. S) had established in 1942 that these strains were able to grow, though badly, in media with, as the sole source of carbon, 0.1 % of glucose which had been sterilized by autoclaving in the complete medium.

In view of Stanier's results these experiments were repeated in 1946 shortly after receipt of his publication. It then appeared that it was indeed possible to obtain good growth in media containing the said low glucose concentration also when heat sterilization was applied. Hitherto it had been found necessary either to autoclave the glucose separately in distilled water, or to keep the pH of the medium to be sterilized at a value not exceeding 7.0. However, when the glucose was autoclaved in an even slightly alkaline medium, no development took place. The behaviour of the three strains in media with higher glucose concentrations had not been tested before the present work was undertaken.

### Methods

For the experiments culture tubes containing 9 ml. of a mineral medium with the composition given below, and various concentrations of glucose, were used. The glucose was added as a concentrated solution in distilled water which was separately sterilized, either by Seitz-filtration or by autoclaving for 15 min. at 110°. The figures given below refer to the final concentrations after addition of glucose to the basal mineral medium. (The glucose used was 'Analar', British Drug Houses Ltd.)

Sometimes parallel tubes were set up both with and without the addition of cellulose (as strips of filter paper No. 597, Schleicher & Schüll). As, however, growth was often poorer in the presence of filter-paper than in its absence, the paper strips were as a rule left out in the experiments reported here. The unfavourable effect of the filter-paper may be due to harmful substances in it (Fåhræus, 1947).

The media indicated in Table 1 were used; they proved to be equally satisfactory.

Table 1

| Medium Dubos                         | (g.)  | Medium g                             | (g.)   |
|--------------------------------------|-------|--------------------------------------|--------|
| NaNO <sub>3</sub>                    | 0.05  | NaNO <sub>3</sub>                    | 0.05   |
| K <sub>2</sub> HPO <sub>4</sub>      | 0.1   | K <sub>2</sub> HPO <sub>4</sub>      | 0.1    |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.05  | MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.05   |
| KCl                                  | 0.05  | KCl                                  | 0.05   |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O | 0.001 | CaCl <sub>2</sub> ·6H <sub>2</sub> O | 0.02   |
| Distilled water to 100 ml.           |       | FeCl <sub>3</sub> ·6H <sub>2</sub> O | 0.002  |
|                                      |       | MnSO <sub>4</sub> ·4H <sub>2</sub> O | 0.0002 |
|                                      |       | Distilled water to 100 ml.           |        |

The pH was adjusted to give a value of 7.2 after autoclaving. All cultures were incubated at 30°, unless otherwise stated.

The tubes were inoculated with a loopful up to a few drops of a suspension either of attacked cellulose fibres taken from a young culture on filter-paper, or of the bacteria present in a previous culture in a sugar medium.

### Results

#### *Growth in liquid glucose-containing media*

First the behaviour of four strains towards different concentrations of glucose autoclaved separately in distilled water was studied.

Table 2. *Period necessary for obtaining visible growth of different Sporocytophaga strains in a medium with glucose autoclaved separately*

Four parallel tubes: 2 without, 2 with filter-paper.

| Strain                  | Glucose concentration (%) |      |     |         |          |
|-------------------------|---------------------------|------|-----|---------|----------|
|                         | 0.0                       | 0.05 | 0.1 | 0.2     | 0.5      |
|                         | Period (days)             |      |     |         |          |
| ' <i>C. globulosa</i> ' | 2-3                       | 3    | 4-5 | 6-9 (i) | 9-14 (i) |
| 3                       | 2                         | 2    | 3   | 5       | 6        |
| H                       | 3-4                       | 2-3  | 2-5 | 5       | 6        |
| D                       | 2                         | 2    | 4   | 5       | 5-6      |

i = growth occurred only in some tubes.

As shown in Table 2 the results were essentially the same for all strains, in that in all cases the development was markedly retarded above a certain glucose concentration. The results confirm those obtained previously (Fåhræus, 1941, 1947). '*Cytophaga globulosa*' seemed to be the most sensitive strain, but

it also finally grew in the highest concentration, although somewhat irregularly. The slightly better growth of this strain than in earlier experiments (cf. Fåhræus, 1947, p. 54) may be due to the use of a different medium. The medium used in the experiments reported here contained nitrate as a source of nitrogen, while most earlier experiments were carried out with ammonia as a nitrogen source. There were also certain other differences, but the decomposition of cellulose in the absence of glucose was unaffected by this change in the composition of the medium.

Subsequently, the growth of '*C. globulosa*' and strain H in various concentrations of filtered glucose was compared with that in a medium with glucose autoclaved separately. A Jena glass filter (G 5 on 8) was used for the filtration. The results are summarized in Table 8.

Table 8. *Period necessary for obtaining visible growth of two Sporocytophaga strains in media with filtered or separately autoclaved glucose*

| Strain         | Treatment of glucose | Substrate    |               |     |       |       |
|----------------|----------------------|--------------|---------------|-----|-------|-------|
|                |                      | Filter-paper | Glucose (%)   |     |       |       |
|                |                      |              | 0.05          | 0.1 | 0.2   | 0.5   |
|                |                      |              | Period (days) |     |       |       |
| 'C. globulosa' | Filtered             | 2-3          | 3-4           | 6-9 | 9 (i) | —     |
|                | Autoclaved           |              | 5             | 6-9 | —     | —     |
| H              | Filtered             | 2            | 2-3           | 5   | 5     | 6     |
|                | Autoclaved           |              | 2             | 4-6 | 5 (i) | 6 (i) |

— = no growth in 21 days.

i = growth only in some tubes.

From these results it is quite clear that, at least under the conditions of the experiment, the filtered glucose also was inhibitory, in agreement with previous results (Fåhræus, 1947).

In other experiments it was found that strains D and W showed the same inhibition by glucose, independently of its pretreatment. An experiment in flasks with a shallow layer of liquid in order to secure good aeration, did not lead to a diminution of the lag phase in glucose media.

An experiment was made with strain H to investigate whether cultivation at 25° would allow better growth in different concentrations of Seitz-filtered glucose than at 30°. Inoculation was restricted to one loopful of a suspension of attacked filter-paper. Within 3-7 days growth had begun in all tubes with filter-paper only, and in those with 0.1 % glucose, whilst in tubes with 0.5 and 1.0 % glucose no turbidity could be observed. However, after 16 days, growth became visible in the first of these tubes; within the next few days development started in more tubes. Thus 24 days after inoculation development was apparent in four out of six tubes with 0.5 and 1.0 % glucose incubated at 25° and in three out of fourteen tubes incubated at 30°. Thirty days after inoculation growth started in one more of the latter tubes. The other tubes did not show visible growth up to the time the experiment was discontinued, at 41 days. Once growth had become apparent, it was abundant within a few days.

These results show that in order to start growth in 0.5 and 1.0 % glucose an incubation temperature of 25° is more favourable than 80°, although development in cellulose media is much more rapid at the latter temperature. As a rule it takes 5–6 days before growth becomes visible at 25° as compared with 8 days at 80°. These results were repeatedly confirmed; Table 4 summarizes

Table 4. *Effect of incubation temperature on time needed for visible growth of Sporocytophaga strain H in filtered glucose*

| Temperature<br>(° C.) | Substrate    |             |     |
|-----------------------|--------------|-------------|-----|
|                       | Filter-paper | Glucose (%) |     |
|                       |              | 0.5         | 1.0 |
|                       |              | Time (days) |     |
| 80                    | 8            | —, —, 24    | —   |
| 25                    | 6            | 10, 10      | 20  |

— = no growth in 28 days.

one of these experiments. Here again good growth in 0.5 and 1.0 % glucose occurred only after a long delay. Obviously, in those tubes in which growth finally took place the cells of the inoculum were alive, but unable to grow during a lag phase which lasted for many days. We therefore conclude that during this period adaptation to the high glucose concentration takes place, as previously suggested (Fåhræus, 1941, 1947). That in the experiments of Table 4 the lag was so much longer than in those summarized in Tables 2 and 3 may be ascribed to differences in strength of the inoculum. Earlier observations (Fåhræus, 1947) support this view, which was further confirmed in an experiment in which tubes with 1.0 % glucose were very heavily inoculated with attacked filter-paper taken from a culture with cellulose only; then growth usually was visible even after only 5 days at 80°. However, this experiment did not exclude the possibility that growth factors were introduced into the medium with the heavy inoculum and were really responsible for the observed shortening of the lag. But the experiment recorded in Table 5 appears to rule out this possibility.

Table 5. *Number of days necessary for obtaining visible growth of 'Cytophaga globulosa' in glucose media on addition of: (1) a thick suspension of living cells; (2) the same suspension boiled; (3) the same after Seitz filtration*

2, 3 and control inoculated with a normal amount of bacteria.

| Suspension | Substrate    |             |     |
|------------|--------------|-------------|-----|
|            | Filter-paper | Glucose (%) |     |
|            |              | 0.2         | 0.5 |
|            |              | Time (days) |     |
| 1          | 2            | 4           | 4   |
| 2          | 3            | —           | —   |
| 3          | 3            | —           | —   |
| Control    | 3            | —           | —   |

— = no growth in 21 days.



If the time required for adaptation is indeed responsible for the long lag period observed after inoculating from a cellulose culture, it would be expected that subculturing from a culture already grown in a glucose medium would not show any growth retardation.

Both for strain H and for strain W it was found that on continued transfer in media with the same high glucose concentration (0.5 or 1.0 %) the first growth was already manifest after 3 days' incubation at 30°. This period is considerably shorter even than that observed in experiments in which very heavy inocula from cellulose cultures were used.

Besides adaptation to high glucose concentrations as such, it appeared that adaptation to a particular concentration of the sugar had taken place. In some experiments at least, in which tubes with 0.1, 0.5 and 1.0 % glucose were inoculated from a young culture in a medium with 1.0 % glucose, it was obvious that growth occurred much more readily in the tube with the corresponding sugar concentration, i.e. 1.0 %.

Although the lag of cellulose-grown bacteria in media with high concentrations of glucose is considerably shorter at 25° than at 30°, for continuous growth in glucose media 30° is a more favourable temperature than 25°. This difference between the optimal temperatures for adaptation and for growth was established for all the strains tested, viz., H, W and D.

Cultures of *Sporocytophaga* growing in liquid glucose-containing media show some remarkable features. The first evidence of growth is nearly always the appearance of small yellowish granules at the surface of the sediment. One day later the liquid has become turbid, and often small floccules appear on the glass wall. Whereas filter-paper is practically only attacked near the surface of the medium, growth in glucose media is not restricted to the surface. Sometimes the turbidity is uniform throughout the medium; in many tubes, however, growth is granular and uneven, and both on the glass wall and in the liquid there occur small colourless or yellowish floccules, as described by Stanier (1942*b*). In old cultures there is always a thick layer of bacteria on the bottom of the tube, and the supernatant liquid becomes more or less clear.

The aerophilic character of the cells is clearly demonstrated by the heavy growth that occurs when the bacteria succeed in maintaining themselves on the surface of the medium. Pl. 1, fig. 4 demonstrates how under these conditions a thick pellicle is formed at the surface; when filter-paper is present this occurs more readily.

The larger floccules sometimes present on the glass walls were about 1 mm. diameter and could easily be picked from the medium, and examined microscopically on a hollow-ground slide. After crushing, the material appears to be composed of both rods and microcysts. These colonies resemble the 'stars' described by Stapp & Bortels for growth on a cellulose medium. In our case, however, the dimensions were much larger.

Pl. 1, fig. 5 shows bacteria obtained from a liquid medium containing 1 % glucose. Contrary to its state in cells from cultures of the same age grown on cellulose, the chromatin is not evenly dispersed in the cells, but is localized;

dividing rods, and the transition forms between rod and microcyst, are very frequent. The rods are somewhat inflated, and the microcysts are larger than in normal cultures. As these differences might be due to suboptimal conditions in the liquid medium owing to insufficient air supply, cells grown in the yellow ring at the meniscus of the water surface were also examined. They were however very similar, though less inflated. Also in bacteria grown in a liquid medium with 0.1 % glucose the localization of the chromatinic material was rather conspicuous, but the cells were not inflated.

*Growth on glucose agar*

Since growth was good in a liquid medium with relatively high glucose concentrations, it was attractive to investigate the behaviour of the strains on nutrient agar containing filtered glucose. Stanier remarked that *Sp. myxococcoides* developed scantily, if at all, on mineral agar with 1.0 % glucose, and that development on plates was regular only when the sugar concentration did not exceed 0.2 %. He suggested that the addition of glucose to the melted agar at 50°, just before pouring the plates, might cause sufficient decomposition of the sugar to affect adversely the subsequent development of the bacteria. Harmsen (1946) describes the formation of distinct colonies of strains of *Sporocytophaga* spp. on cellulose agar containing 0.1 % or 0.25 % glucose.

In our attempts to cultivate *Sporocytophaga* on glucose agar we started from both cellulose and glucose cultures. We prepared plates of the same composition as Dubos's nutrient medium (Table 1) with 1.5 % agar that had been carefully washed with distilled water for several days. The agar was cooled to 37°, mixed with a Seitz-filtered glucose solution at the same temperature, and the plates poured immediately.

In several experiments strain H grown in a 1 % glucose medium was streaked on plates containing 1 % glucose. Many colonies were seen after 5 days at 30°. After 12 days the largest colonies had a diameter of 2–3 mm. (Pl. 1, fig. 6). They were yellow-orange in colour, very slimy, and in the centres of the colonies darker parts of a peculiar shape were visible, all colonies being of the same character.

In another experiment, with the same strain, streaks were made from a heavy suspension of decomposed filter-paper on to 0.1 and 1 % glucose agar. On the latter no growth occurred, but on 0.1 % glucose agar a great many colonies were seen after 5 days. The colonies were rather faint in colour and less thick and slimy than those on 1 % glucose described earlier. In this case too all colonies were of the same type (cf. Pl. 1, fig. 7).

In several other experiments it appeared that growth occurred more readily when the glucose plates were inoculated from cultures containing the corresponding concentration of glucose, once more suggesting that adaptation to the given concentration of the sugar had taken place.

It should be stressed that development on glucose agar was not always successful; in several cases growth failed completely notwithstanding that under the same conditions in other instances good growth had been obtained. We have no explanation for this.

The microscopical appearance of bacteria grown on 0.1 % glucose agar can be seen in Pl. 1 fig. 8.

From strain W grown in 0.1 and in 1 % glucose media, streaks were made on 0.1 and in 1 % glucose agar. In addition, transfers were made from a heavy suspension of decomposed filter-paper to plates with both sugar concentrations. With the exception of the plates inoculated from the cellulose culture, in all cases colonies developed after 7 days' incubation at 30°. On these plates two types of colonies could be distinguished (cf. Stanier, 1942*b*), one of which might be called a typical rough form, the other a more or less smooth form. The rods from the smooth colonies were rather long and swollen, those of the rough colonies giving a more normal picture. A cellulose medium inoculated from a rough colony supported a more rapid growth than a similar medium inoculated from a smooth colony.

In a second experiment with the same strain streaks were made from a heavy suspension of attacked filter-paper on 0.1 and 1 % glucose agar. On the latter, no growth occurred, but on 0.1 % glucose agar many colonies were visible after 5 days incubation. Here too, rough (cf. Pl. 1, fig. 9) and smooth colonies could be distinguished, the smooth being somewhat smaller than in the first experiment.

#### *Growth on mannose, fructose and xylose*

Stanier's *Sp. myxococcoides* did not attack mannose, whereas the two strains studied by Fåhræus (1947) both did so. The remaining strains H, W and D were now tested in this respect, and appeared to grow well on filtered mannose (0.1, 0.5, 1.0 %). Nevertheless, here too there was a lag of about the same duration as in the experiments with glucose.

Cultures grown in 1 % glucose medium were examined to see whether they would develop without delay in a medium containing the same concentration of mannose. Transfers were made from young cultures of strains H and W grown in 1 % glucose to nutrient media with 1 % mannose and with 1 % glucose; the tubes were incubated at 30°. Growth was visible after 3–4 days in both media.

Experiments were carried out to investigate the suitability of fructose and xylose as a substrate for the strains H, W and D. The strains of *Sporocytophaga* spp. investigated by Stanier (1942*b*) and by Fåhræus (1947) did not grow on any of these sugars. However, since xylan is consumed by several strains of *Sp. myxococcoides* (Fuller & Norman, 1942, 1943) these strains might grow on xylose. Tubes containing 1 % filtered fructose or xylose were inoculated with cultures of the strains H, W and D grown on 1 % glucose. No growth occurred even after 28 days incubation. Control tubes containing 1 % glucose inoculated at the same time showed visible growth already after 3–4 days. This experiment was repeated several times with the same negative results. Hence it is probable that for the strains tested fructose and xylose cannot act as a substrate.

Further experiments supported this view. Tubes of nutrient media provided with a strip of filter-paper and tubes which contained in addition 1 % fructose

or xylose were inoculated with a heavy suspension of attacked filter-paper. In the tubes without sugar the attack on the filter-paper was manifest after 2 days incubation; after 8 more days the strips disintegrated at the surface of the medium. In the tubes containing fructose or xylose a coloration of the filter-paper became visible after 2-4 days, and 3-5 days later here too the paper was disintegrated at the surface. The nutrient medium remained perfectly clear in all cases. These observations seem to indicate that neither fructose nor xylose had been consumed; the disintegration of the filter-paper was, however, slightly delayed by the presence of these sugars. The three strains H, W and D gave similar results.

In another experiment we tested whether the strains could be adapted to growth on fructose or xylose; small pieces of filter-paper that had been attacked in a medium containing 1 % fructose or 1 % xylose failed to cause turbidity after transfer to nutrient media with the same concentrations of these sugars, whereas turbidity was visible after 4 days in analogous cultures in glucose media.

*Growth on glucose autoclaved in the nutrient medium*

Glucose in culture media is liable to decomposition by heat treatment, the more so in a slightly alkaline medium. The intensity of the yellowish tinge formed in glucose-containing media during autoclaving seems to run parallel to their inhibiting action upon certain micro-organisms. In earlier, unpublished investigations, one of us (A. K. S.), however, observed that 0.1 % glucose could be autoclaved in the nutrient medium without any unfavourable effect on the growth of *Sporocytophaga*, if the pH was not allowed to exceed 7.0 during the heating. Growth on such media was as good as in media containing 0.1 % glucose sterilized either by Seitz-filtration, or by heating in distilled water. When, however, the pH was 7.2 or higher during autoclaving a marked inhibition of growth usually occurred.

These experiments were extended for strain H to media with 0.5 and 1.0 % glucose. A duplicate series of tubes was prepared containing either one of these glucose concentrations or a strip of filter-paper. In one series the pH was adjusted before autoclaving to 7.0, in the other to 7.6. The tubes were inoculated with cultures grown on filter paper, and incubated at 30° without readjustment of the pH. The filter-paper in the glucose-free tubes in both series was attacked after 3 days. Thus incubation at the different pH values did not appear to influence the development of the bacteria. In tubes with 0.5 % glucose at pH 7.0 growth was visible after 12 days, whereas in those at pH 7.6 no growth was visible even after 21 days. Irrespective of the pH, there was no growth in any of the tubes with 1.0 % glucose in this period. Thus in the sugar-containing media autoclaved at pH 7.6 growth was impossible, whereas for the same media autoclaved at pH 7.0 results were comparable with those obtained in media to which separately sterilized glucose was added.

## DISCUSSION

Earlier investigations of one of us (G. F.) showed that bacteria of the 'cytophaga'-type when decomposing cellulose start by hydrolysing this polysaccharide so that finally glucose is the nutrient on which these organisms thrive. However, all available evidence suggests that in these circumstances the concentration of the glucose always remains very low. In this connexion it is instructive that our experiments leave no doubt that the bacteria in question do not grow immediately in media with relatively high glucose concentrations (0.5–1.0%). Evidently the presence of glucose in these concentrations initially paralyses the normal metabolism, for if cellulose is simultaneously present it is not attacked either, during the lag period. Apparently a certain adaptation is necessary before normal metabolism becomes possible again. Then glucose is attacked preferentially, and the cellulose breakdown starts only when the greater part of the glucose has been consumed.

These results are at variance with the observations of Stanier, who reported that his strains of *Sporocytophaga myxococcoides* grew readily in media with a glucose concentration of 0.5% or even much higher. At present no explanation can be offered for this contrast, and we can only assume a specific difference between his strains and ours.

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Figs. 1-9



EXPLANATION OF PLATE

- Fig. 1. *Sporocytophaga myxococcoides*, strain H. Grown on filter-paper; 5 days old; stained with Hucker's methyl violet stain for 20 min.;  $\times 800$ .
- Fig. 2. Idem, strain W.
- Fig. 3. Idem, strain D.
- Fig. 4. Strain H in 1 % glucose medium. Pellicle of bacteria at the surface of the medium and bacterial sediment at the bottom. Incubation period from left to right: 28, 18 and 11 days.
- Fig. 5. Strain H grown in 1 % glucose medium; 5 days old; Hucker's methyl violet stain;  $\times 800$ .
- Fig. 6. Colonies of strain H on 1 % glucose agar. Inoculated from liquid medium with 1 % glucose; 12 days old. Diameter plate 8 cm.
- Fig. 7. Colony of strain H on 0.1 % glucose agar. Inoculated from cellulose culture; 7 days old;  $\times 12$ .
- Fig. 8. Strain H grown on 0.1 % glucose agar. Inoculated from cellulose culture; 8 days old;  $\times 800$ .
- Fig. 9. Rough colony of strain W on 0.1 % glucose agar. Inoculated from cellulose culture; 7 days old;  $\times 12$ .

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## The Relationship between Micro-organisms and Soil Aggregation

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**SUMMARY:** The physical condition of soil is improved by adding readily decomposable organic material. Microbial cells and metabolic products affect soil structure by binding loose soil particles into water-stable aggregates.

Experimentally, the relative aggregating power of pure cultures of micro-organisms was as follows: fungi > actinomycetes and a few gum-forming bacteria > many gum-producing bacteria > yeasts, proactinomycetes, and many bacteria; the last three groups did not improve aggregation. Fungal hyphae entangled soil particles into stable aggregates; weaker crumbs were formed by the frailer threads of actinomycetes. A few bacterial strains produced gums capable of glueing soil into water-stable aggregates, but the majority of bacterial slimes were almost useless because they remained water-soluble after drying. The cementing properties of these gums was not improved by treatment with H or Ca ions. Bacterial gums stabilized the aggregates produced from completely dispersed soils and kaolin, but not those formed with bentonite or ferric hydroxide. The pH value of the soil played a very minor part in influencing the aggregation produced by pure cultures of micro-organisms or even by soil inoculum.

Mixed cultures of fungi or of actinomycetes gave slightly better aggregation than pure cultures, but neither capsulated nor non-capsulated bacteria in mixtures gave better results than single strains. More complex mixtures containing fungi, actinomycetes and bacteria gave good aggregation when all micro-organisms were compatible, but poor results when antagonistic bacteria inhibited the growth of either fungi or actinomycetes. The fair aggregation obtained with soil inoculum was reproduced in the laboratory by inoculating sterilized soil with complex mixtures of micro-organisms.

A study was made of the relative merits of glucose, starch, blood, yeast, fungal mycelium, straw, clover and farmyard manure for encouraging aggregation by mixtures of fungi, actinomycetes and bacteria.

Aggregates bound by mycelia did not last long because the hyphae were decomposed by bacteria. The temporary improvement of soil structure after the addition of organic materials can be partly explained by the action of microbes, but the permanent crumb structure of many soils must be due mainly to other causes.

Many of the older countries of the world have succeeded in maintaining large populations only by conserving the physical condition of the soil with rotational cropping or alternate husbandry. It has long been known that the maintenance of soil friability depends on periodically supplying readily decomposable organic matter, and that ley farming methods are not only profitable, but improve the structure of all types of soil. Ley farming might be adopted more readily if more were known of the mechanism whereby soil crumbs are restored by grass roots and other organic materials. The task of combating erosion is closely linked with the problem of restoring the crumb structure of soil. This paper deals with some aspects of the role of micro-organisms and organic matter in improving the aggregation of soil.

The biological factors influencing soil structure have been reviewed in a recent publication of the Commonwealth Bureau of Soil Science (1948). Geltser (1936, 1937, 1943) found that readily decomposable organic materials temporarily improved the crumb structure of soils, whereas materials more resistant to decomposition were less beneficial. She considered that stable aggregates were formed only after fungal hyphae had been replaced by bacteria, which in turn were lysed and formed organic cements. Martin (1945) inoculated sterilized soil containing sucrose with pure cultures of fungi, actinomycetes and bacteria and found that the hyphae of *Cladosporium* spp. and gum from *Bacillus subtilis* greatly increased aggregation, but that actinomycetes and other bacteria were less effective. Subsequently Martin (1946) isolated polysaccharides from several bacteria, including fructosans from *Bacillus subtilis* and *Azotobacter indicum* and dextrans from *Leuconostoc dextranicus* and two unidentified strains, and found them to be better crumb cements than casein or lignin. These polysaccharides were slowly decomposed by several micro-organisms. In pure culture studies McCalla (1945, 1946) classified the aggregating power of soil micro-organisms as follows: fungi and a few gum-forming bacteria > actinomycetes > yeasts > most bacteria.

Most workers have tried to interpret field phenomena by experiments with pure cultures of micro-organisms, often specially selected because they possessed good aggregating properties. Little account was taken of the fact that the soil contains numerous organisms all competing with one another; and no attempt was made to estimate the relative proportion of micro-organisms beneficial, useless and harmful to aggregation, that are present in soil after the addition of organic materials.

#### EXPERIMENTAL METHODS

*Micro-organisms.* Throughout the text the micro-organisms are designated by a prefixed letter and a number. Thus B stands for a bacterium, A for an actinomycete, and F for a fungus. The reference number, name, a brief description and place of origin of the cultures most commonly used are as follows.

B2 *Bacillus mycoides*; Haywards Heath.

B8 *Micrococcus* sp.. Gram-positive, white mucoid colony; Haywards Heath.

B35 *Aerobacter* sp., slimy colony; Rothamsted.

B45 *Achromobacter* sp., Gram-negative, gummy colony; Haywards Heath.

B50 *Sarcina* sp., Gram-positive, yellow waxy colony; Rothamsted.

B68 *Achromobacter* sp., Gram-negative, gummy colony; Rothamsted.

B91 *Bacillus* sp., rubbery colony; Rothamsted.

B168 *Pseudomonas fluorescens*, non-mucoid colony; London brick earth.

B169 *Bacillus polymyxa*, gelatinous colony; from Prof. Kluver, Delft.

A8 *Actinomyces* sp., white powdery colony; Haywards Heath.

A16 *Actinomyces coelicolor*, purple colony; Rothamsted.

A20 *Micromonospora* sp., brown gelatinous colony; Haywards Heath.

F7 *Absidia glauca*; Haywards Heath.

F27 *Aspergillus nidulans*; London brick earth.

F51 *Trichoderma lignorum*; Rothamsted.

Most of the work was carried out with three English soils: (1) Rothamsted allotment grey clay loam, of pH 6.9; (2) Haywards Heath light, grey-brown, sandy loam, podsol of pH 4.7; (3) Woburn light brown, sandy loam, podsol, limed to pH 6.7.

*Production of microbial gums.* Bacteria and fungi were cultivated for 20 days at 25° in shallow layers of the following medium: 20 g. glucose, 5 g. peptone, 2 g.  $\text{NH}_4\text{NO}_3$ , 0.5 g.  $\text{K}_2\text{HPO}_4$ , 0.25 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g.  $\text{CaCl}_2$ , 10 ml. soil extract, made up to 1 l. with distilled water. The bacterial cells were removed by centrifugation and the fungal hyphae by filtration. Bacterial gums were precipitated from the resulting fluid by 3 volumes of ethanol, collected on a Buchner filter, washed with ethanol, dispersed in water and dialysed to remove salts. Proteins were removed by repeatedly shaking with chloroform and the gums reprecipitated with 3 volumes of ethanol, washed with ethanol and dried at room temperature.

Crude fungal gums were prepared by fragmenting the fungal hyphae in a Waring Blender, treating them with sodium carbonate and trypsin overnight to digest proteins and boiling the residue left after filtration with 0.1 N-NaOH. The gums were precipitated by freezing, washed with water then ethanol and dried at room temperature.

*Cultivation of micro-organisms in soil.* The soils were air-dried, crushed, passed through a 2 mm. sieve and 50 g. lots weighed into large test-tubes, 7 in. long and 1½ in. diameter. It was found that a 2 mm. fraction gave better results than a 1 mm. fraction because better aeration was obtained. After plugging, the tubes of soil were dry sterilized for at least 4 hr. at 150°. Insoluble foodstuffs, such as oat-straw and dried blood, were ground and 0.5 % mixed with the soil before heat sterilization. Soluble nutrients, such as glucose, peptone and salts, were dissolved in sufficient water to bring the 50 g. of soil to a water content just up to field capacity. These solutions were tubed and autoclaved separately. The sterile soils were inoculated by adding a loopful of micro-organisms first to the tube of sterile nutrient solution, mixing thoroughly, and pouring the contents aseptically into the tube of soil. Ultimately the soil contained 0.5 % sugar, 0.036 % peptone or 0.014 %  $\text{NH}_4\text{NO}_3$ , 0.01 %  $\text{K}_2\text{HPO}_4$ , 0.005 %  $\text{CaCl}_2$  and 0.005 %  $\text{MgSO}_4$ . In all experiments tubes were prepared in triplicate for each treatment so that for all standard errors quoted  $n=2$ . The tubes were incubated at 25° and losses due to evaporation restored by adding sterile water at intervals of approximately 20 days. At the end of incubation the plugs were removed, and the soils were dried in an oven for 48 hr. at 50°. After this they could be stored without deterioration until aggregate analyses were carried out.

*Aggregate analysis of soils.* At first the soils were analysed both for micro-aggregates and for macro-aggregates, but when it was found that the results were parallel, only the macro-aggregates were estimated. Usually 50 g. of soil were used for each determination. Soils that had become compacted during

incubation were gently broken by hand into lumps of about 1.5 cm. Then, instead of allowing the moisture gradually to soak through by capillarity, a more vigorous action was obtained by flooding the lumps or crumbs of soil in a Petri dish, where they were soaked for 24 hr. This relatively long period of soaking was chosen to give closer approximation to field conditions, where soils are often moist for weeks on end. It was found that many bacterial gums were not fully hydrated after only a few hours soaking, so that a false value for their cementing power was obtained unless they were soaked for 24 hr.

Micro-aggregates were determined by transferring the wet soil to a measuring cylinder, filling up to the 1 l. level, shaking end over end 20 times, floating a Bouyoucos hydrometer in the suspension, and reading after 30 sec. settling. A temperature correction was made and this gave the amount of suspended non-aggregated material  $< 0.07$  mm. in diameter, from which the weight of micro-aggregates and sand  $> 0.07$  mm. in diameter was found by difference. Allowance was made for the sand  $> 0.07$  mm. by determining the amount present after completely dispersing the soil by shaking overnight with sodium oxalate solution.

Macro-aggregates  $> 1$  mm. in diameter were determined by transferring the soaked soil to a sieve with 1 mm. holes and hand-sieving under water until all fine particles had passed through. This usually required sixteen to twenty strokes. The aggregates and sand retained on the sieve were dried at  $50^{\circ}$  and weighed. In any one experiment the amount of sand was considered to be constant, so no deduction was made.

## EXPERIMENTAL RESULTS

Myers & McCalla (1941) counted the number of micro-organisms in soil containing sucrose and found that maximum aggregation occurred after the bacterial population had reached its peak. They concluded that bacterial products, rather than cells, were responsible for aggregation.

*Aggregate formation in unsterilized soil.* A test for correlation between the bacterial and fungal population and the degree of aggregation was made as follows. Two 5 kg. samples of Haywards Heath soil in glass pots were treated respectively with 0.5 % dried blood and 0.5 % soluble starch, and one sample was untreated. The pots were moistened to field capacity with a mineral solution containing soil inoculum, and incubated at  $25^{\circ}$ . At intervals over a period of 86 days, micro- and macro-aggregation was estimated, and at the same time dilution plate counts of fungi were made on acid glucose peptone agar, and of bacteria and actinomycetes on peptone soil-extract agar. The pH was determined on all soil samples. On two occasions the bacterial numbers and fungal lengths were determined by the Jones & Mollison (1948) direct microscopic method.

The percentages of micro-aggregates almost paralleled the percentages of macro-aggregates throughout the whole period of incubation (Fig. 1). Both the control and the blood treatment produced an initial decrease in the amount of aggregation. After 8 days the percentage aggregation of untreated soil

remained fairly constant except for minor fluctuations. The percentage of micro-aggregates in soil containing blood improved fairly steadily throughout the whole experiment, but the percentage of macro-aggregates decreased somewhat at the 52nd day. The starch-treated soil aggregated rapidly and aggregation reached a peak between the 17th and 26th days, but then declined.

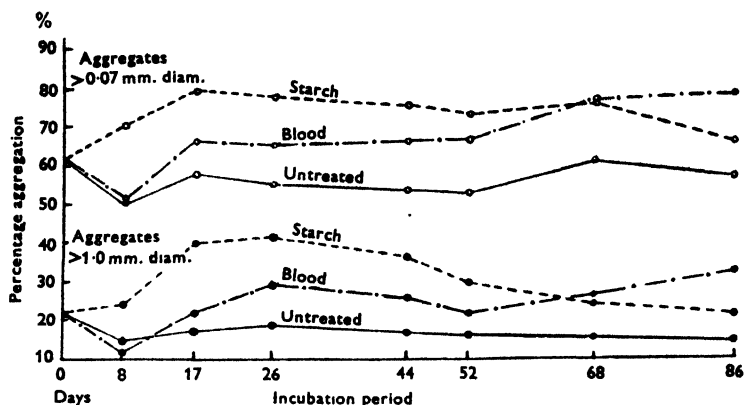


Fig. 1. Influences of starch and blood in aggregation of Haywards Heath soil.

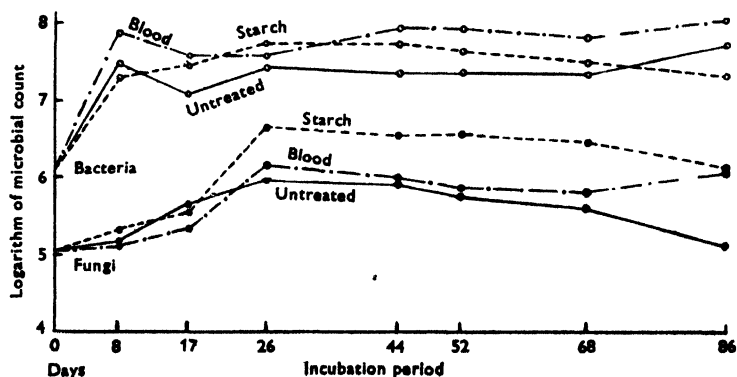


Fig. 2. Bacterial and fungal counts of Haywards Heath soil treated with starch and blood.

There was an initial sharp rise in the bacterial plate count in all three treatments (Fig. 2). In general, blood produced the highest counts and even after 86 days the peak was not reached. With starch the bacterial count reached a maximum after 26–44 days, then declined slightly. Throughout most of the incubation period the untreated soil contained fewer bacteria than the two treated soils. There were no obvious differences in the types of bacteria and actinomycetes attributable to the various treatments.

The fungal population as estimated by plate counts was always less than that of bacteria. The fungal count of all three treatments rose steadily until the 26th day, remained fairly constant until the 68th day, and then fell in the untreated and starch treated soils. During most of the experiment starch gave

higher counts than either blood or untreated. There were no obvious differences in the types of fungi isolated from starch, blood or untreated soils.

On the two occasions when direct microscopic counts were made by the method of Jones & Mollison (1948), the figures greatly exceeded the number found by dilution plating. At the start of the experiment all treatments contained 12.7 m. of fungal hyphae and 1350 million bacteria per g. soil. On the 17th day starch, blood and untreated soil contained 96.2, 49.5 and 25.8 m. of fungal hyphae and 9500, 11,330 and 3.470 million bacteria respectively per g. soil.

The pH of the untreated soil remained between 4.7 and 5.3 during the whole period. Blood-treated soil became neutral on the 26th day and remained thus until the ammonia began to nitrify after 52 days, when the pH fell to 4.7. Contrary to expectation, soil containing starch gradually became less acid with incubation and finally reached a pH of 6.1. Doubtless microbial activity was influenced by fluctuations in soil pH, but it is doubtful whether aggregation was greatly affected by pH alone, since artificial adjustment of the reaction with alkali from pH 4.7 to 7.5 had no appreciable effect on macro-aggregation.

It is difficult to draw from plate counts any definite conclusions about the relative importance of bacteria and fungi in improving the structure of the differently treated soils. It is doubtful whether either population can be even approximately estimated by dilution plating. When breaking the clods of soil by hand it was observed, however, that good aggregation was invariably associated with a visibly luxuriant growth of fungi throughout the soil.

After 17 days' incubation, the lengths of fungal mycelium were correlated with the degree of aggregation (see p. 252), but neither plate nor microscopic counts of bacteria closely paralleled the increase in aggregation during the earlier stages of incubation, perhaps because such counts included a majority of organisms having no aggregating power. Hence it was decided to isolate various microbes from Haywards Heath and Rothamsted soils and to test their aggregating power in pure culture.

#### *Aggregation of soil by pure cultures*

Seventy-five strains of bacteria, twenty-one of actinomycetes, five of pro-actinomycetes, five of yeast and fifty of fungi were cultured in tubes of sterile Rothamsted allotment soil (< 2 mm.) containing 0.5 % of glucose, 0.036 % of peptone and the usual mineral salts. After 20 days at 25° the soils were subjected to macro-aggregate analysis by wet sieving. Table 1 classifies the seventy-five bacterial strains according to their ability to produce fair (13-16 g.), poor (10-13 g.) or no (7-10 g.) aggregation of 50 g. soil. Observations on the presence of mucoid colonies on glucose peptone agar, the formation of chains of cells and Gram-staining are also given. In general bacteria were not good aggregators of soil despite the fact that most of them grew well. Twenty-two of the strains gave fair aggregation, twenty-five gave poor, while twenty-eight did not aggregate the soil at all. There was no consistent correlation between

the degree of aggregation and the production of mucoid colonies on agar, cell morphology or the Gram-reaction. The best aggregator from Haywards Heath soil was B 45, which produced 15.02 g. crumbs/50 g. soil, while the best from Rothamsted was B 68, which produced 14.86 g. crumb/50 g. soil. Both organisms were species of *Achromobacter* that produced very gummy colonies. Many other strains also produced gums which bound the soils when dry or after a short period of soaking, but after 24 hr. in water most of these gums redispersed and the aggregates were slaked.

Table 1. *Characters of some soil bacteria as related to their ability to aggregate soil in pure culture*

| Aggregation | Mean weight of aggregates > 1 mm./50 g. soil (g.) | Total no. of strains | No. of strains having the characters shown |            |                           |              |            |    |
|-------------|---|----------------------|--|------------|---------------------------|--------------|------------|----|
|             |   |                      | Consistency of colonies on agar            |            | Cell grouping of bacteria |              | Gram stain |    |
|             |   |                      | Mucoid                                     | Non-mucoid | Chains                    | Single cells | +          | -  |
|             |   |                      |  |            |                           |              |            |    |
| Fair        | 13-16   | 22                   | 10   | 12         | 2                         | 20           | 8          | 14 |
| Poor        | 10-13   | 25                   | 21   | 4          | 2                         | 23           | 10         | 15 |
| None        | 7-10  | 28                   | 12   | 16         | 10                        | 18           | 16         | 12 |
| Totals      |   | 75                   | 43   | 32         | 14                        | 61           | 34         | 41 |

Table 2. *Influence of actinomycetes, proactinomycetes and yeasts on soil aggregation*

| Type of micro-organism | Aggregation | Mean weight of aggregates > 1 mm./50 g. soil | No. of strains |
|------------------------|-------------|--|----------------|
| Actinomycetes          | Good        | 21-29  | 7              |
| Actinomycetes          | Fair        | 13-21  | 12             |
| Actinomycetes          | Poor        | 10-13  | 2              |
| Proactinomycetes       | None        | 7-10   | 5              |
| Yeasts                 | None        | 7-10   | 5              |

Subsequently many other bacterial strains from various sources were tested and only two gave better results than B 45 and B 68, viz. *Bacillus subtilis* (Geoghegan's strain) and *B. polymyxa* (B169). The aggregating powers of actinomycetes, proactinomycetes and yeasts are summarized in Table 2.

Usually actinomycetes were found to be better aggregators than bacteria, but proactinomycetes and yeasts did not cement the soil at all. Seven vigorously growing actinomycetes gave good aggregation (21-29 g.), twelve of them produced fairly stable crumbs (13-21 g.), while two species of *Micro-monospora* formed only a few stable aggregates (10-13 g.). It was observed that strains which produced numerous waxy spores prevented the soil from being properly wetted so that amyl alcohol had to be added to the water. The best aggregator was strain A 8 (28.46 g.) which also inhibited wetting of the soil with water. Many other actinomycetes from other soils were tested and the majority gave good to fair aggregation. Table 3 classifies the fungi according to their influence on soil aggregation and to the type of mycelium produced

on glucose peptone agar. In general pure cultures of fungi produced far better aggregation than all other micro-organisms tested. The best strains were vigorous growers, producing woolly mycelia on both soil and agar, e.g. species of *Absidia*, *Mucor*, *Rhizopus*, *Chaetomium*, *Fusarium* and *Aspergillus*. Slightly poorer results were obtained by slower growing strains which gave a more prostrate growth on soil or agar, e.g. some species of *Penicillium*, *Cladosporium*, *Alternaria* and *Rhizoctonia*. Fair aggregation only was obtained by a very

Table 3. *Influence of fungi on soil aggregation*

| Aggregation | Mean weight<br>of aggregates<br>> 1 mm./50 g.<br>soil (g.) | Total<br>no. of<br>strains | Growth on agar<br>(no. of strains) |           |
|-------------|--|----------------------------|------------------------------------|-----------|
|             |  |                            | Woolly                             | Prostrate |
| Excellent   | 37-45  | 21                         | 18                                 | 3         |
| Very good   | 29-37  | 15                         | 10                                 | 5         |
| Good        | 21-29  | 11                         | 3                                  | 8         |
| Fair        | 13-21  | 3                          | 0                                  | 3         |
|             | Totals   | 50                         | 31                                 | 19        |

slow-growing unidentified fungus and by *Oospora lactis* and a *Monilia* sp. whose hyphae tended to break up into fragments. Again it was observed that vigorous sporers like penicillia and aspergilli impeded wetting of the soil. The best aggregator isolated from Haywards Heath soil was *Absidia glauca* (F7) (44.62 g.), but similar strains were isolated subsequently from many other types of soil.

It was rather surprising that most bacteria were less effective than actinomyces or fungi, since other workers had found that bacterial gums were very effective in glueing soils into water-stable crumbs. In most cases these workers carried out aggregate analysis after wetting the soils for a shorter time than the 24 hr. needed to dissolve water-soluble gums; and they used specially selected strains of bacteria without stating whether they were abundant in field soils. Before dismissing bacteria as unimportant aggregators of soil enriched with organic materials, some further experiments were carried out to find whether the addition of liquid cultures to soil gave better results than growing the bacteria *in situ*.

#### *Aggregation by bacterial liquid cultures*

Previously the bacterial strains had been divided according to their ability to produce fair, poor, or no aggregation, and for the present experiment eight strains were selected from each of the three groups. They were grown for 20 days at 25° in shallow liquid medium containing 2.0 % glucose, 0.5 % peptone and the usual salts, shaken and their turbidity and viscosity estimated. Samples (15 ml.) were mixed with 50 g. of Rothamsted allotment soil (< 1 mm.) and after drying at 50° macro-aggregate analyses were carried out in the usual way. During soaking it was noticed that some of the treated soils wetted slowly, while others wetted readily. Table 4 classifies the bacteria according to their aggregating power, their influence on the wettability of soil, and the turbidity and viscosity of the cultures in the liquid medium.



In most cases the aggregation produced by liquid cultures was practically the same as that produced by bacteria grown in soil, with the exception that two strains formerly classified as fair and poor aggregators respectively were reclassified as poor and non-aggregators respectively. There was no perfect correlation between cementing power of the bacteria and any of their other properties, but better aggregation tended to be associated with poor wettability of soil and with high turbidity and viscosity of liquid cultures.

Table 4. *Influence of various bacterial properties on the aggregation and wettability of soil treated with liquid cultures*

| Aggregation | Weight of aggregates<br>1 mm./50 g.<br>soil (g.) | Total<br>no. of<br>strains | No. of strains with characters shown |      |                                |     |                                |     |
|-------------|--|----------------------------|--------------------------------------|------|--------------------------------|-----|--------------------------------|-----|
|             |  |                            | Waterproofing<br>action on soil      |      | Turbidity of<br>liquid culture |     | Viscosity of<br>liquid culture |     |
|             |  |                            | Low                                  | High | High                           | Low | High                           | Low |
| Fair        | 12-15  | 7                          | 2                                    | 5    | 5                              | 2   | 5                              | 2   |
| Poor        | 9-12   | 8                          | 2                                    | 6    | 6                              | 2   | 2                              | 6   |
| None        | 6-9  | 9                          | 4                                    | 5    | 6                              | 3   | 3                              | 6   |
| Totals      |  | 24                         | 8                                    | 16   | 17                             | 7   | 10                             | 14  |

Six of the same strains from each of the three aggregation classes were grown in fifteen different media, all containing 0.5 % of peptone as the source of nitrogen, the usual salts and 2.0 % of various metabolites including one pentose, three hexoses, three disaccharides, one trisaccharide, three polysaccharides, one salt of an organic acid, two hexahydric alcohols and one peptone. During incubation it was noticed that the turbidity and viscosity of the cultures varied greatly, depending on the nutrients present. In the majority of cases glucose gave the best growth and the highest viscosity, but galactose, maltose and mannitol were often almost as good. The liquid cultures were added to soil as previously and the stability of the aggregates was determined (Table 5). It is evident that the stability of the aggregates depended on the bacterial culture medium. Metabolic products from glucose produced the most stable aggregates. Strains originally classified as fair aggregators also usually produced stable crumbs when cultivated in galactose, maltose and mannitol. Some poor aggregators also gave a few fairly stable crumbs when grown in mannitol and maltose. With few exceptions the strains from the non-aggregating class produced no stable aggregates from any media, although B35 and B163 both from this last group yielded fairly stable aggregates with mannitol and peptone respectively.

Two factors might influence the cementing power of strains grown in different media. Some nutrients might favour the production of larger quantities of gum than others. Judging by the various amounts of gum precipitated by ethanol from some of the media this is almost certainly true. It is also possible that the chemical composition of the gums and their glueing properties might depend on the source of carbon, but this point was not investigated. However, most bacteria isolated from soil do not produce very stable aggregates.

*Properties and effects of microbial gums*

In view of the large amounts of uronide carbon found in soils by Fuller (1946, 1947 *a*, 1947 *b*) an attempt was made to correlate the physical and chemical properties of various microbial gums with their cementing power after mixing 0.5 % of gum with sifted Rothamsted allotment soil (<1 mm.). Gums from

Table 5. *Influence of bacterial foodstuff on stability of soil aggregates*

Effect of groups of six bacterial strains originally classified by their behaviour with glucose as

| No. of strains giving aggregates that were ...<br>Metabolite: | Fair   |          | Poor   |          | None   |          |
|---|--------|----------|--------|----------|--------|----------|
|   | Stable | Unstable | Stable | Unstable | Stable | Unstable |
| Xylose  | 1      | 5        | 0      | 6        | 0      | 6        |
| Glucose   | 6      | 0        | 6      | 0        | 0      | 6        |
| Laevulose   | 1      | 5        | 1      | 5        | 0      | 6        |
| Galactose   | 6      | 0        | 2      | 4        | 0      | 6        |
| Sucrose   | 3      | 3        | 1      | 5        | 0      | 6        |
| Maltose   | 6      | 0        | 3      | 3        | 0      | 6        |
| Lactose   | 2      | 4        | 2      | 4        | 0      | 6        |
| Raffinose   | 0      | 6        | 0      | 6        | 0      | 6        |
| Dextrin   | 2      | 4        | 1      | 5        | 0      | 6        |
| Soluble starch  | 1      | 5        | 1      | 5        | 0      | 6        |
| Inulin  | 2      | 4        | 1      | 5        | 0      | 6        |
| Calcium gluconate   | 0      | 6        | 2      | 4        | 0      | 6        |
| Mannitol  | 5      | 1        | 4      | 2        | 1      | 5        |
| Sorbitol  | 0      | 6        | 1      | 5        | 0      | 6        |
| Peptone   | 0      | 6        | 1      | 5        | 1      | 5        |

four bacteria and two fungi were prepared by the methods given previously (p. 238). In addition, fungal hyphae of strain F27 were composted for 20 days at 25° after inoculating with soil suspension and a gum was isolated from the sludge by the technique used for preparing bacterial polysaccharides. A gum from the fulvic acid fraction of soil humus was isolated by the method of Forsyth (1947). Sodium  $\alpha$ -humate was extracted from Rothamsted allotment soil by the method used by Waksman (1938). A few of the physical and chemical properties of these eight gums are summarized in Table 6. For comparison the properties of a typical soil humate are also given. Only two gums were insoluble in water, viz. from F7 and F27, and they produced of course very stable aggregates when their hot alkaline solutions were added to soil. Of the remaining six gums, all were soluble in aqueous solutions of NaOH, HCl and CaCl<sub>2</sub>, except for a rather impure gum isolated from F27 compost, which was precipitated by calcium salts. Half these polysaccharides were flocculated by FeCl<sub>3</sub>; all were precipitated from aqueous solution by 75 % ethanol. The difference in behaviour of soil humate from most of the gums is very striking. Evidently the other constituents in humus either mask the properties of the polyuronides also present, or else polysaccharides different from those studied are involved.

There was no clear correlation between the cementing powers of the gums and the particular properties studied.

Most of the bacterial gums when mixed with soil gave only poor to fair aggregation because most of the gums diffused out into the water during sieving operations. Treatment with  $H^+$  or  $Ca^{++}$  ions did not improve matters because the gum films were not precipitated by these ions. It was thought that

Table 6. *Some properties of gums from bacteria, fungi, decomposing fungi, and soil*

| Test or reagent          | <i>Achromo-<br/>bacter</i> sp.<br>(B45) | <i>Achromo-<br/>bacter</i> sp.<br>(B68) | <i>B. poly-<br/>myxa</i><br>(B169) | <i>B. sub-<br/>tilis</i> *           | <i>Absidia<br/>glauca</i><br>(F7) | <i>Asper-<br/>gillus<br/>nidulans</i><br>(F27) | Decom-<br>posing<br>F27 | Soil<br>gum†                   | Na-z-<br>humate<br>(Rotham-<br>sted) |
|--------------------------|---|---|------------------------------------|--------------------------------------|-----------------------------------|--|-------------------------|--------------------------------|--------------------------------------|
| Cold $H_2O$              | Sol.                                    | Sol.                                    | Sol.                               | Sol.                                 | Insol.                            | Insol.   | Sol.                    | Sol.                           | Sol.                                 |
| Boiling $H_2O$           | Sol.                                    | Sol.                                    | Sol.                               | Sol.                                 | Insol.                            | Insol.   | Sol.                    | Sol.                           | Sol.                                 |
| 0.1 N-NaOH               | Sol.                                    | Sol.                                    | Sol.                               | Sol.                                 | Sol.-hot,<br>insol.-cold          | Sol.-hot,<br>insol.-cold                       | Sol.                    | Sol.                           | Sol.                                 |
| 0.1 N-HCl                | Sol.                                    | Sol.                                    | Sol.                               | Sol.                                 | Insol.                            | Insol.   | Sol.                    | Sol.                           | Insol.                               |
| 0.1 N- $CaCl_2$          | Sol.                                    | Sol.                                    | Sol.                               | Sol.                                 | Insol.                            | Insol.   | Sl. sol.                | Sol.                           | Insol.                               |
| 0.1 N- $FeCl_3$          | Insol.                                  | Sol.                                    | Insol.                             | Sol.                                 | Insol.                            | Insol.   | Sl. sol.                | Sol.                           | Insol.                               |
| 75 % ethanol             | Insol.<br>(floc.)                       | Insol.<br>(powder)                      | Insol.<br>(strings)                | Insol.<br>(powder<br>and<br>strings) | Insol.<br>(floc.)                 | Insol.<br>(floc.)                              | Insol.<br>(floc.)       | Insol.<br>(floc.)              | Sol.                                 |
| Sugars in<br>hydrolysate | Glucose                                 | Glucose                                 | Glucose<br>mannose                 | Laevulose                            | —                                 | —  | —                       | Glucose<br>xylose<br>arabinose | —                                    |
| Uronic acid<br>group     | Present                                 | Present                                 | Present                            | —                                    | —                                 | —  | —                       | Present                        | Present                              |
| Aggregation<br>(%)       | 34.10<br>± 1.42                         | 12.28<br>± 0.98                         | 53.48<br>± 2.34                    | 90.80<br>± 2.12                      | 97.26<br>± 2.76                   | 96.51<br>± 3.26                                | 65.19<br>± 2.22         | 8.20<br>± 0.86                 | 27.70<br>± 1.88                      |

\* Obtained from M. J. Geoghegan, Jealott's Hill Research Station

† Obtained from W. G. C. Forsyth, Macaulay Institute.

merely mixing them with soil paste might not give adequate contact between the colloidal micelles of gum and those of clay. Consequently an attempt was made to separate the clay particles by water molecules and to insert the polysaccharides between them.

#### *Stability of aggregates formed by co-precipitation*

Suspensions (4 %) of Na-bentonite, Na-kaolin and freshly prepared ferric hydroxide were shaken overnight to disperse them completely. Similar concentrations of Rothamsted surface soil and subsoil were dispersed with sodium oxalate. Colloidal solutions of gums from *Achromobacter* spp. (B45, B68) and *B. polymyxa* (B169) were added to these suspensions of clays and soils in a concentration of 0.02 % by weight of gum. The mixtures of ferric hydroxide and gums began to coagulate and settle almost immediately but the other suspensions remained stable. Co-precipitates were formed by adding 0.05 N- $CaCl_2$  and allowing the floccules to settle overnight. The supernatant was decanted and tested for gums by adding 8 volumes of ethanol and looking for a precipitate after 24 hr. No gums were detected from ferric hydroxide treatments, and

only traces were obtained from other treatments. The co-precipitates were dried at 50°, the flakes were resoaked for 48 hr. and wet-sieved. The weight of aggregates from 10 g. of material are given in Table 7. Ferric hydroxide gave extremely stable aggregates both in the presence and absence of bacterial gums. Bentonite flakes swelled and slaked more when gums were added than when they were absent. The reverse was the case with kaolin, Rothamsted surface soil and subsoil, with which the gums improved aggregation.

Table 7. *Stability of aggregates formed by co-precipitating clays or soils with bacterial gum*

| Clay or soil              | Bacterial gum used                                  |              |              |             |
|---------------------------|---|--------------|--------------|-------------|
|                           | (Mean weight (g.) of aggregates > 1 mm./10 g. soil) |              |              |             |
|                           | B 45  | B 68         | B 169        | Untreated   |
| Fe(OH) <sub>3</sub>       | 10.02 ± 0.26  | 10.00 ± 0.20 | 10.03 ± 0.29 | 9.96 ± 0.27 |
| Na-bentonite              | 8.51 ± 0.19   | 8.30 ± 0.33  | 8.68 ± 0.48  | 9.63 ± 0.35 |
| Na-kaolin                 | 6.85 ± 0.25   | 6.96 ± 0.37  | 7.72 ± 0.41  | 4.87 ± 0.13 |
| Rothamsted soil (0-4 in.) | 8.90 ± 0.46   | —            | —            | 7.53 ± 0.50 |
| Rothamsted soil (18 in.)  | 9.31 ± 0.23   | —            | —            | 6.25 ± 0.18 |

In natural soils it is possible that over a long period of time micelles of bacterial gum might diffuse in between platelets of clay and thereby cement them better. This would presuppose that the bacterial gums were fairly resistant to microbial attack. Martin (1945, 1946) found that bacterial polysaccharides were slowly attacked by soil organisms. Gums isolated from B 45, B 68, B 169 and *B. subtilis* (Geoghegan) certainly lose their viscosity after inoculation with micro-organisms. Ensminger & Gieseking (1942) found that proteins were protected from enzymic digestion by clay and it is possible that adsorbed polyuronides might also survive microbial attack.

#### *Influence of pH on aggregation by pure cultures*

An experiment was planned to determine the effect of soil pH on the aggregation brought about by different micro-organisms.

Haywards Heath soil of pH 4.7 was chosen for this experiment, because unlike Rothamsted allotment soil it contained no free lime and its reaction could be changed readily by the addition of KOH or H<sub>3</sub>PO<sub>4</sub>. After adjustment to pH values of 3.5, 4.7, 6.0, 7.2 and 8.5, sterile soil samples were treated with 0.5 % of glucose and 0.086 % of peptone and minerals, then separate samples inoculated respectively with bacteria B 2 and B 45 from the fair-aggregation class, bacteria B 35 and B 91 from the poor cementing group, and bacteria B 50 and B 163 from the non-aggregation class. In addition an actinomycete, A 3, two fungi, F 7 and F 27, and Rothamsted soil inoculum were also used in the experiment. After 3 weeks at 25° aggregate analyses were made. The results for the bacterial treatments may be summarized by saying that B 2 gave no significant difference in aggregation at the acid pH values of 3.5, 4.7 and 6.0 and the alkaline value of 8.5, but better aggregation at pH 7.2;

B45 produced no stable aggregates at pH 8.5, but fairly stable crumbs at all other values; B35 did not grow at pH 3.5, and B35 and B91 produced little or no aggregation at any reaction; and finally B20 failed to grow at pH 8.5, and B50 and B168 formed no stable crumbs throughout the entire pH range. In contrast to the poor performance of bacteria, A3 formed fairly stable crumbs equally well at all pH values between 4.7 and 8.5, but was killed by pH 3.5. The fungi F7 and F27 were checked a little at the extreme acid values, but produced very good aggregation at most other pH values. Soil inoculum gave results intermediate between the actinomycete and the fungi. Soil pH did not affect the aggregation of sterile soil. The aggregation of uninoculated soil receiving sterile glucose, peptone and salts was no different from that receiving sterile water only.

Evidently the pH value plays a very minor part in influencing the aggregation produced by pure cultures of micro-organisms or even by soil inoculum. This is in agreement with field experience, where the addition of readily decomposable organic matter improves the structure of all soil types irrespective of pH value. It is realized, however, that some organisms may be dominant at one pH and other types at another pH; but the resultant effect on structure is much the same.

Tests with pure cultures can hardly be expected to elucidate effects obtained in the field, so experiments were made with mixed cultures of micro-organisms.

#### *Aggregation of soil by mixed cultures*

Two bacteria (B45 and B169), two actinomycetes (A3 and A16) and two fungi (F7 and F27) were added singly and in combinations of two, three and six organisms into 50 g. of sterilized Rothamsted allotment soil (< 2 mm.), together with 0.5 % of glucose, 0.036 % of peptone and minerals; sterile soil and soil inoculum were also included in the treatments. After 3 weeks at 25° the soil aggregates were analysed.

As already observed, pure cultures of the fungi gave very good aggregation, the actinomycetes produced good aggregation, while single cultures of bacteria formed only a fair weight of crumbs. Pairs of fungi or actinomycetes produced significantly more aggregates than single organisms, possibly because they mutually assisted one another; but the pair of bacteria was no better than strain B169 alone. When a fungus or an actinomycete was grown in association with a bacterium, there was a small increase in aggregation which in some combinations attained significance. Possibly the bacterial gums were prevented by the network of hyphae from diffusing into the water during soaking of the soil. There was also a small but insignificant increase in effect in mixtures of a fungus with an actinomycete.

Mixed cultures of three micro-organisms consisting of a pair of bacteria, of actinomycetes or of fungi together with a third organism sometimes produced a significant improvement as compared with the same pair or the same three components growing separately. Mixtures containing fungi always produced more aggregates than mixed cultures containing only actinomycetes and bacteria.

The effects of a bacterium, an actinomycete and a fungus alone, and of mixtures of these organisms are shown in Pl. 1, fig. 1.

The best aggregation of all was obtained when all six organisms were grown together. In view of this fact it was rather surprising to find that soil inoculum, containing numerous microbial types, formed much fewer aggregates.

*Influence of inhibitory bacteria on soil aggregation*

The unexplained behaviour of soil inoculum stimulated further investigation of the compatibility of mixtures of other microbial strains. Bacteria capable of inhibiting the growth of fungi and actinomycetes growing on agar were easily found, and it was suspected that the anomalous behaviour of soil inoculum might be due to the presence of such bacterial antagonists. Accordingly, an experiment was conducted to find whether inhibitory bacteria could prevent the formation of aggregates by fungi and actinomycetes.

Two bacteria, B8 and B163, antagonistic to the growth of F7 and A3, were inoculated singly and in combinations into the usual amount of sterile Rothamsted soil enriched with glucose, peptone and minerals. After 20 days at 25° the soils were analysed by wet sieving; the figures are given in Table 8.

Table 8. *Influence of inhibitory bacteria on aggregation of soil by actinomycetes and fungi*

| Micro-organisms used | Mean weight of<br>aggregates<br>> 1 mm., 50 g. soil<br>(g.) |
|----------------------|---|
|                      | — — — — —   |
| B8                   | 9.58 ± 0.71   |
| B163                 | 8.32 ± 1.12   |
| A3                   | 19.98 ± 0.43  |
| F7                   | 36.27 ± 1.16  |
| B8 + B163            | 8.46 ± 0.27   |
| B8 + A3              | 11.10 ± 0.20  |
| B8 + F7              | 13.84 ± 1.79  |
| B163 + A3            | 10.75 ± 0.49  |
| B163 + F7            | 15.17 ± 1.39  |
| A3 + F7              | 39.95 ± 0.91  |
| B8 + A3 + F7         | 24.20 ± 0.32  |
| B163 + A3 + F7       | 20.78 ± 1.12  |
| B8 + B163 + A3 + F7  | 18.63 ± 0.74  |
| Soil inoculum        | 21.54 ± 0.46  |
| Sterile              | 8.77 ± 0.14   |

B8 and B163, grown singly, did not significantly improve soil structure. On the other hand, A3 in pure cultures produced good aggregation, F7 also gave excellent results, and the pair together gave the best effect of all. The presence, in mixtures, of the inhibiting bacteria B8 and B163, whether combined with one or two other organisms, always decreased the aggregation. This is seen in Pl. 1, fig. 2 which shows the effect of growing B163, A3 and F7 in various combinations.

Such inhibition can hardly be explained by competition for food because free glucose was often found at the end of the experiment. It seems more likely that it was due to the production by the bacteria of antibiotic substances which hindered the growth of the fungus and the actinomycetes. This suggested imitating the effect of soil inoculum on aggregation by using very complex mixtures of bacteria, actinomycetes and fungi, some of which were perfectly compatible and others antagonistic. By using selected micro-organisms it was possible to use not only glucose as foodstuff but also more complex materials such as plant and animal products. It was hoped that this would lead to a better understanding of the improvement in soil structures obtained when crop residues, green manure crops, or ley pastures were ploughed in.

*Influence of mixed cultures and complex organic materials on aggregation*

Various organic materials (0.5 %) were added to soil. As examples of readily available carbohydrates, glucose and soluble starch were used in conjunction with 0.036 % of peptone and minerals. Blood was tried as a readily decomposable protein. Oat-straw, with a low nitrogen content and a moderately high percentage of lignin, was added as an example of a typical crop residue. Red clover with a fair nitrogen content was used as representing a common green manure crop. The nitrogen supply was augmented by adding 0.014 % of  $\text{NH}_4\text{NO}_3$ . Rotted farmyard manure was included as a material that had become humified through composting. It is probable that dead bacterial and fungal cells serve as nutrients for other soil micro-organisms; so killed and dried yeast cells and mycelium of *Aspergillus niger* were included in the trial. Each of these organic materials was tested in Rothamsted clay loam, and glucose, straw and clover were also tested in Woburn sandy loam. Soils with each added material were divided into sets inoculated respectively with twenty-two fungal, thirty-three actinomycete and sixty-six bacterial strains and with a mixture of the above 121 strains. A set with soil inoculum and a sterile control were also included. All inocula contained micro-organisms capable of decomposing cellulose, chitin, starch, pectin, agar, lignin, resins, fats, proteins and sugars. Soil inoculum was included for comparison. Macro-aggregate analyses were carried out after 3, 6 and 9 weeks at 25°.

In comparable treatments the heavier textured Rothamsted soil aggregated better than the light soil from Woburn, especially with oat-straw and red clover. Apart from this difference the various inocula and organic materials behaved similarly in both soil types. The mean aggregating influence of different groups of organisms utilizing the same foodstuffs ran in the following order: fungi > soil inoculum = fungi + actinomycetes + bacteria > actinomycetes > bacteria > none. This suggested that soil inoculum could be closely imitated by using a complex microbial mixture containing numerous fungi, actinomycetes and bacteria, some of which were compatible and others incompatible. In Rothamsted soil the relative effect of the organic materials varied greatly with the inoculum and length of incubation. In Woburn soil the superior effect of the readily

available glucose was striking. Pl. 2, fig. 3 shows the influence of the various inocula on Rothamsted soil supplied with glucose.

Previous workers have found that soil structure was restored more rapidly by readily decomposable materials than by resistant materials; this finding is supported by the good effects of glucose, yeast and starch. It appears that good crumb structure can be achieved only when maximum microbial activity is promoted by a plentiful supply of easily digested food, especially materials which encourage fungi, actinomycetes and gum-forming bacteria.

#### *Disaggregation of soil stabilized by fungal hyphae*

An experiment was designed to find which organisms caused disaggregation of a soil that had been mechanically stabilized by fungal hyphae.

Fifty portions of Rothamsted allotment soil (<2 mm.) were sterilized in percolation funnels plugged at the bottom with glass wool and on the top with cotton wool. A sufficient volume of medium containing spores of the fungi, F7 or F27, was added to bring the soil moisture to field capacity and to provide 0.5 % of glucose, 0.014 % of  $\text{NH}_4\text{NO}_3$  and the usual minerals. The fungi were allowed to grow for 3 weeks at 25° until they had stabilized all the soil aggregates and the residual soluble metabolites and products were then leached out aseptically with Hoagland & Arnon's complete nutrient medium sterilized and diluted. Some of the tubes of soil were autoclaved to kill the fungi, while others were left unheated. Six sets were then inoculated respectively with mixtures containing ten different fungi, ten strains of actinomycetes, seven, fifteen and thirty of selected bacteria and soil inoculum. A sterile control was also included. The mixtures of fungi, actinomycetes and bacteria had previously been tested for ability to grow in a shallow liquid medium containing only sterilized fungal fragments and minerals. The bacterial inocula contained organisms capable of digesting a wide variety of substances including cellulose, chitin, agar, starch, sugars, resins, butter-fat and proteins. The inoculum containing seven bacteria included two *Cytophaga* spp. *Pseudomonas fluorescens*, *Bacillus mycoides*, an *Achromobacter* sp., and two unidentified strains capable of digesting agar and butter-fat. Inocula comprising fifteen and thirty strains included these seven strains together with various isolates from soil previously enriched with dead fungal fragments. Macro-aggregate analyses were carried out at intervals over a period of 8 weeks.

Under sterile conditions dead hyphae of F27 continued to bind the soil together throughout the experimental period. Growth from the inoculum containing the ten fungi caused no loss of structure during the period of the experiment, probably because dead fungal threads were replaced by living hyphae, so that the soil remained aggregated. With the ten actinomycetes, however, aggregation declined, probably because the fungal mycelia were replaced by weaker threads of actinomycetes. Treatment with seven bacterial strains and soil inoculum caused a marked deterioration of structure even after 2 weeks at 25°. This effect was accentuated when fifteen and thirty bacterial strains were used. Evidently the products of bacterial decomposition of the



fungus F27 were not as effective in aggregating the soil as the original fungal hyphae. Pl. 2, fig. 4 shows the effect of the various inocula on soil aggregates originally stabilized by F27. Similar results were obtained with living hyphae of F27, but here the treatments gave smaller losses in aggregation than with dead hyphae. In contrast to the results from fungus F27, the other soil stabilizing fungus F7 proved to be very resistant to bacterial attack. Fungi and actinomycetes certainly grew on the dead mycelium of F7, but as before, only the actinomycetes caused any disaggregation. Microscopic examination of the soil inoculated with numerous bacteria showed that they were growing but were not decomposing the cell walls of the hyphae of F7, so that soil aggregation remained practically unchanged over a period of 8 weeks. Similarly, soil inoculum caused no disaggregation until after 6 weeks' incubation.

*Estimated influence of micro-organisms on aggregation of field soil*

An attempt was made to calculate the amount of macro-aggregation that might be attributed to fungal hyphae and to gum-forming bacteria in Rothamsted allotment soil that had been cultivated for many years.

Pure cultures of *Absidia glauca* (F7) and *Aspergillus nidulans* (F27), growing in sterilized Rothamsted soil enriched with glucose, formed 242 and 374 m. of hyphae/g. of soil as determined by the method of Jones & Mollison (1948) and they entangled 96.5 and 80.3 % respectively of the soil into stable aggregates. The length of fungal hyphae, found in fresh Rothamsted allotment soil was 38.8 m./g. The length of hyphae should thus aggregate 15.5 % of the soil, if they behaved like species F7 and 8.4 % if like species F27. The fresh soil actually contained 38 % of aggregates, an appreciable fraction of which might hence be attributed to the effect of fungi.

A connexion between fungal mycelium and aggregation is also shown in the data previously quoted from Haywards Heath soil incubated for 17 days with the addition respectively of blood, starch and untreated. Here the percentage of macro-aggregates ( $a$ ), though very different with the various treatments, yet shows a regular relationship to the lengths of mycelium ( $m$ ) in which  $a = 0.3m + K$ , where  $K$  is a constant of value 9.5 %. This is shown in the following figures.

| Treatment | $m$  | Percentage aggregation $a$ |       |
|-----------|------|----------------------------|-------|
|           |      | Calculated                 | Found |
| Untreated | 25.8 | 17.2                       | 17.0  |
| Blood     | 49.5 | 24.3                       | 22.2  |
| Starch    | 96.2 | 38.4                       | 39.0  |

The estimate of aggregation attributable to slimy bacteria was based on a test with strain B45 and thus assumes this strain to be representative. It also assumes that the proportion of slimy bacteria was the same in the natural field soil as it was amongst plate colonies derived from a suspension of that soil.

On these assumptions the glueing power of the native bacteria accounts for only about 2 % of aggregation out of a total of 38.0 % in the field soil. On these

calculations the combined effect attributable to mycelial threads and to bacterial gums can account for less than half of the macro-aggregates found in fresh Rothamsted soil. While it is true that microbial threads and gums could largely account for the increased temporary aggregation of soils supplied with readily decomposable organic matter, it is evident that existing fungal and actinomycete hyphae plus capsulated bacteria cannot account for the aggregation of natural Rothamsted soil to which fermentable material has not recently been added. One must thus conclude that the formation of more permanent crumbs found in certain soils must be due to other cementing substances, e.g. colloidal clay, humus or resistant gums produced by micro-organisms no longer visible.

The author wishes to express his gratitude to the Sir Benjamin Fuller Travelling Scholarship Trust for making this research possible and his sincere thanks to Dr H. G. Thornton, F.R.S., for helpful suggestions and criticism. Thanks are also due to Miss Mabel Dunkley for preparing the typescript and to the technical staff of the Department of Soil Microbiology for valuable assistance.

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## EXPLANATION OF PLATES

## PLATE 1

Fig. 1. Influence of pure and mixed cultures of micro-organisms on aggregation. The bacterium was B45, the actinomycete, A3, and the fungus, F7.

Fig. 2. Influence of inhibiting bacterium B163 on aggregation by actinomycete A3 and fungus F7.

## PLATE 2

Fig. 3. Influence on mixed inocula on aggregation of soil enriched with glucose.

Fig. 4. Microbial disaggregation of soil previously stabilized by hyphae of fungus F27. 1, sterile; 2, ten strains of fungi; 3, ten strains of actinomycetes; 4, seven strains of bacteria; 5, fifteen strains of bacteria; 6, thirty strains of bacteria.

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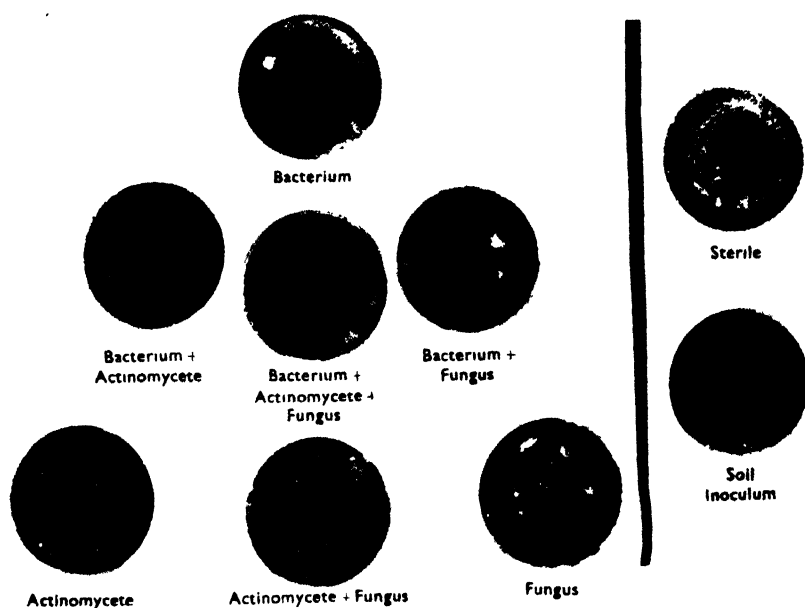


Fig. 1

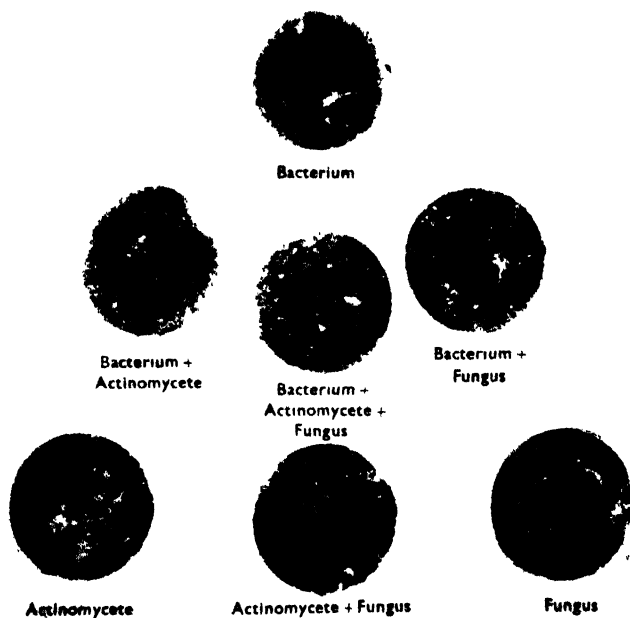


Fig. 2

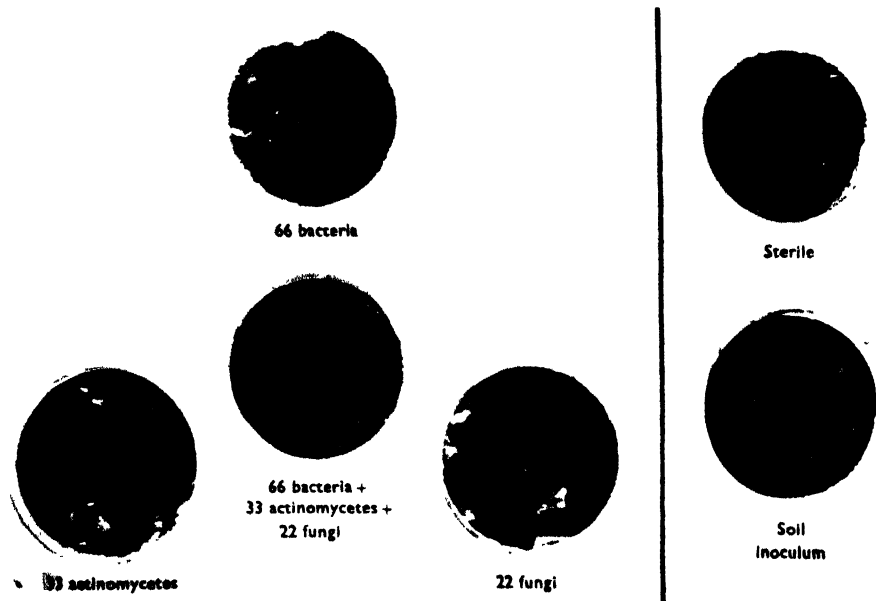


Fig. 3

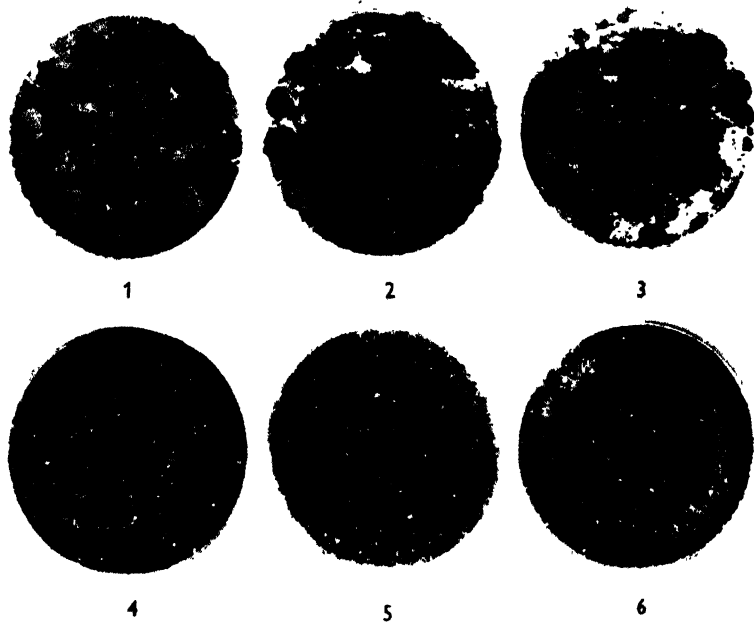


Fig. 4

## The Lecithinase of *Bacillus cereus* and its Comparison with *Clostridium welchii* $\alpha$ -toxin

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**SUMMARY:** *Bacillus cereus* and *B. mycoides* produce lecithinases which split lecithin into phosphoryletholine and a diglyceride in the same way as the lecithinase ( $\alpha$ -toxin) of *Clostridium welchii*. These enzymes also possess most of the biological activities associated with *Cl. welchii*  $\alpha$ -toxin, e.g. produce the Nagler reaction and the egg-yolk reaction, lyse red blood cells, and are dermonecrotizing and lethal.

The enzymes are activated by Ca and Mg ions, but inhibited by Na, K,  $\text{NH}_4$  ferrie and Al ions. Optimal enzyme activity requires the presence of Ca ions within a narrow range of concentration of  $1\text{--}4 \times 10^{-3}\text{M}$ . It is interesting that at this concentration of Ca ion lecithin flocculates most readily from its emulsion. The enzyme thus seems to have a maximal affinity for lecithin when the latter adsorbs an optimal amount of Ca ion in reaching its isoelectric point.

Like the *Cl. welchii* lecithinase, the *B. cereus* lecithinase is fairly resistant to heat. The lysis of red blood cells and the hydrolysis of free lecithin by *B. cereus* lecithinase was strongly inhibited by normal sera of all the animals tested. But when lecithin was bound in egg-yolk lipoproteins, its hydrolysis by the enzyme was unaffected by normal serum. Specific serum, on the other hand, was capable of inhibiting the hydrolysis of both free lecithin and protein-bound lecithin. The *B. cereus* and *B. mycoides* lecithinases are immunologically related, but they are not so related to *Cl. welchii* lecithinase.

In a previous communication (McGaughey & Chu, 1948) it was reported that, in the *Bacillus* group, *Bacillus cereus*, *B. mycoides* and *B. anthracis* were able to produce an egg-yolk reaction similar to that caused by *Clostridium welchii*  $\alpha$ -toxin. In the case of *Cl. welchii*  $\alpha$ -toxin, the reaction was found to be due to the action of a lecithinase which splits lecithin into phosphoryletholine and diglyceride (Macfarlane & Knight, 1941). A similar type of lecithinase has now been demonstrated in *B. cereus* and *B. mycoides*, and the activity also runs parallel with the yolk reactivity. In view of the close correlation between the lecithinase activity and the haemolytic, necrotic and lethal activities of *Cl. welchii*  $\alpha$ -toxin, it was considered interesting to see whether the lecithinases obtained from these new sources and causing the same biochemical reaction on the substrate lecithin also possess the biological properties of the *Cl. welchii* lecithinase.

### MATERIALS AND METHODS

**Cultures.** Although many strains of *B. cereus*, *B. mycoides* and *B. anthracis* were used, for most of the work the enzyme was prepared from a strain of *B. cereus* (N.C.T.C. 945).

**Preparation of lecithin.** Lecithin was prepared and purified by the methods of Maclean & Maclean (1927) and Welch (1945). Fresh egg yolks were extracted with several changes of ethanol. The ethanol extract was evaporated to dryness *in vacuo* and taken up in a small volume of ether. Crude phospholipids were precipitated

from the ethereal solution with excess of acetone. The precipitate was taken up again in ether and kept in the refrigerator to allow sphingomyelin and galactolipin to separate out. After centrifuging, the clear ethereal solution was again treated with acetone and the process was repeated three times. Finally, the acetone precipitate, which contained chiefly lecithin and cephalin, was taken up in absolute ethanol. After standing in the refrigerator overnight, the cephalin which precipitated was removed by centrifugation. The purification was repeated until the product gave a perfectly clear solution in ice-cold ether or ethanol.

As lecithin is very unstable in air, it was found convenient to make a thick emulsion of the product as soon as it was prepared and to distribute it in fixed quantities, usually 250 mg., in small ampoules. They were then dried from the frozen state and sealed *in vacuo*. Lecithin kept in this form remained apparently unchanged for a long time. For use, the contents of an ampoule were washed out with water and diluted to the required volume. Lecithin kept in this form emulsifies very readily. The product generally contained 1.95–2 % N, 3.75–3.9 % P, 13.8–14.2 % choline with a molecular ratio of P and choline 1:0.95–0.97.

*Preparation of cephalin.* Cephalin was prepared from sheep brain according to Maclean & Maclean (1927). The product contained 3.76 % P but no choline. It was not hydrolysed by *Cl. welchii* lecithinase, and was thus practically free from lecithin.

*Preparation of lipovitellin and lipovitellenin.* Lipovitellin and lipovitellenin, the two egg-yolk lipoproteins, were prepared according to Alderton & Fevold (1945) and Fevold & Lausten (1946). The former contains 16–18 % and the latter 35–40 % of phospholipins, of which about 80 % is lecithin.

*Chemical analysis.* Total P was determined photoelectro-colorimetrically by Brigg's method after ashing with  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ . N was estimated by the micro-Kjeldahl method and Ca by precipitation as oxalate and permanganate titration. For the estimation of choline, a modification of Beattie's reineckate method (Glick, 1944) was used.

*Clostridium welchii*  $\alpha$ -toxin. The *Cl. welchii*  $\alpha$ -toxin used in this investigation was a glycerinated preparation containing 2800 lecithinase units (Macfarlane & Knight, 1941) per ml., kindly supplied by Dr van Heyningen (1941*b*).

*Measurement of the lecithinase activity.* Like *Cl. welchii* lecithinase, *B. cereus* lecithinase splits lecithin into acid-soluble phosphorylcholine, and the rate of increase of acid-soluble phosphate also serves as a convenient measure of the enzyme activity. The method employed was essentially the same as that used for *Cl. welchii* lecithinase (Macfarlane & Knight, 1941). The reaction mixtures were made up with 1 ml. 0.05 M borate buffer pH 7.1; 0.1 ml. 0.1 M- $\text{CaCl}_2$  (for *Cl. welchii* lecithinase 0.2 ml. was used); aqueous solution of enzyme and distilled water to 3 ml. After this mixture had been warmed to 37°, 1 ml. of a 2.5 % lecithin emulsion at the same temperature was added from a 1 ml. blow-out pipette to ensure an immediate mixing. The reaction was stopped after 15 min. incubation at 37° by the addition of 1 ml. 20 % trichloroacetic acid, which also flocculated the undecomposed lecithin. The mixture was then filtered through a no. 50 Whatman filter-paper and the total phosphate in the filtrate estimated. The enzyme activity in the following experiments was generally expressed as mg. of acid-soluble phosphate liberated from 25 mg. of lecithin in 15 min. at 37° and one arbitrary unit of lecithinase was defined as that amount of enzyme which under the above conditions would catalyse the production of 0.1 mg. acid-soluble P.

*Measurement of the egg-yolk reaction.* The production of opalescence in egg-yolk extract by the action of lecithinase was measured by a modification of

van Heyningen's (1941*a*) method. The standard conditions finally adopted were as follows: 1 ml. enzyme diluted in water, 1 ml. 0.05 M borate buffer (pH 7.1) and 2 ml. distilled water were put in a test-tube or preferably in the cups of the photoelectric colorimeter, and warmed to 37°; 2 ml. 5% egg-yolk extract at the same temperature were then added. After 15 min. at 37° the turbidity was read in a photoelectric colorimeter fitted with a grey filter.

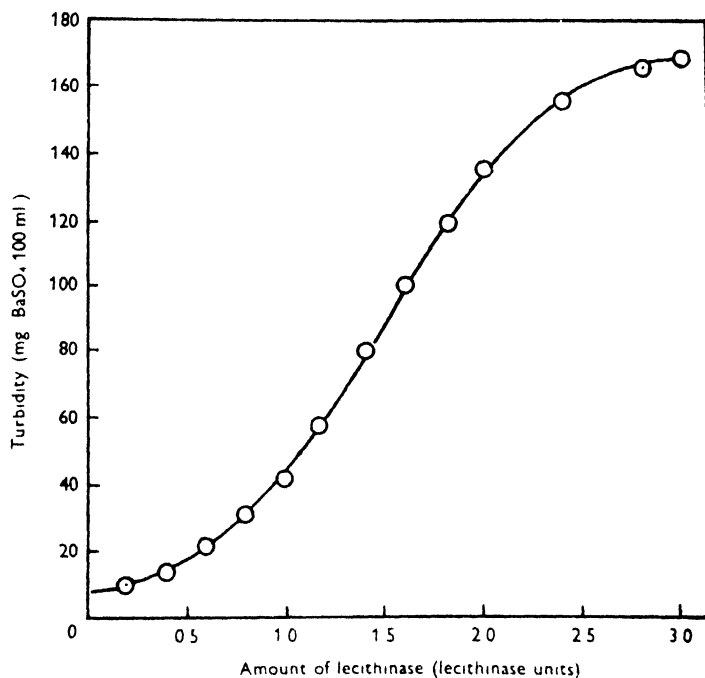


Fig. 1. The effect of lecithinase concentration on development of turbidity in egg-yolk extract. Reaction mixture: amount of lecithinase indicated above, 1 ml.; borate buffer pH 7.1; 2 ml. yolk saline; water to 6 ml.; reaction time 15 min. at 37°.

In the photoelectric colorimeter (King, 1946) the reading ('extinction coefficient  $E$ ' multiplied by 100) is not a linear function of the turbidity, especially when the reading is greater than 30. For this reason the turbidities were read from a standard curve prepared by plotting the colorimeter readings of a series of suspensions of  $\text{BaSO}_4$ . Using this curve the turbidity of a given egg-yolk reaction mixture was expressed in terms of its equivalent  $\text{BaSO}_4$  concentration. The standard  $\text{BaSO}_4$  suspensions were prepared as follows: a stock suspension of  $\text{BaSO}_4$  was prepared by adding 1% (v/v) conc.  $\text{H}_2\text{SO}_4$  to 2.0 ml. 1% (w/v)  $\text{BaCl}_2$  with stirring to a total volume of 100 ml. The resultant suspension was calculated to contain 224 mg. of  $\text{BaSO}_4$ /100 ml. A series of suspensions containing 1–200 mg.  $\text{BaSO}_4$ /100 ml. were then prepared by diluting the stock  $\text{BaSO}_4$  suspension with 1% conc.  $\text{H}_2\text{SO}_4$ .

A dry enzyme preparation containing 3 lecithinase units per mg. was used as the standard for the egg-yolk turbidity estimation. Fig. 1 shows the turbidities produced in 15 min. by 0–3 units of lecithinase under the above



conditions. It can be seen that in the region of from 1 to 2 units, the turbidity is a linear function of the enzyme concentration.

It must be emphasized that under the above standard conditions the final reaction mixture (total volume of 6 ml.), is 0.8 % with respect to NaCl and contains no Ca ion except that present in the egg yolk. In the case of *Cl. welchii* sufficient Ca ion is added to a final concentration of 0.005 M. A slight change in the salt concentration has a great influence on the turbidity produced. As the absolute turbidity produced by a certain amount of enzyme is influenced by a number of conditions which can hardly be identical in two occasions, it is advisable to run a few standards with each set of estimations. When the enzyme to be estimated is present in a complicated medium the standard enzyme preparation must be diluted with the same medium so that the effect of the salts and other substances present in the medium can be eliminated.

*Measurement of haemolytic activity.* Haemolytic activity was measured by modifications of the methods of Herbert (1941) and van Heyningen (1941*b*). Decreasing amounts of haemolysin diluted to a total volume of 2 ml. with borate buffer saline (pH 7.1) containing  $10^{-4}$  M- $\text{Ca}^{++}$  were incubated with 1 ml. of a washed sheep red-cell suspension standardized to contain 20 mg. haemoglobin/ml. (equivalent to c. 6 % cell suspension). After 1 hr. at 37° the reaction was stopped by the addition of 7 ml. of normal saline containing 5 % of normal horse serum, which was found to inhibit strongly the haemolytic activity of *B. cereus*. The unhaemolysed cells were then removed by centrifugation and the percentage haemolysis was estimated from the haemoglobin content of the supernatant fluid in a photo-electric colorimeter fitted with a green filter. The colorimeter readings were transformed directly into percentage haemolysis by reference to a standard curve prepared with a series of standard haemoglobin solutions. This curve was also used for the standardization of the red-cells suspension.

An arbitrary haemolytic (H.U.) unit is defined as the amount of haemolysin causing 50 % haemolysis of 1 ml. suspension of sheep red blood cells containing 20 mg. haemoglobin after 1 hr. at 37°. Fig. 2 shows the degree of haemolysis produced by 0.5–3.5 H.U. of *B. cereus* haemolysin. The shape of the curve is similar to that of streptolysin O (Herbert, 1941) and *Cl. welchii*  $\alpha$ -toxin (van Heyningen, 1941*b*).

## RESULTS

### *Concentration of the enzyme*

A 20 hr. nutrient broth culture of *B. cereus* was used as a source of the enzyme. To the culture filtrate, which generally contained 1–2 units of lecithinase, ammonium sulphate was added slowly with stirring to two-thirds saturation. The precipitate, which contained most of the enzyme, was then redissolved in one-twentieth of its original volume of water. To this solution half a volume of saturated ammonium sulphate solution was added and the resultant dark brown sticky precipitate was centrifuged off. The clear supernatant was dialysed against running tap water for 8 hr., and then against several changes of distilled water at 2° for 24 hr. The dialysed solution was either dried to

a solid *in vacuo* or concentrated by dialysing against 50% aqueous glycerol. The latter preparation retained its enzymic activity unchanged for over  $1\frac{1}{2}$  years. The glycerinated solution generally contained 50–80 lecithinase units per ml. and the dry preparation about three lecithinase units per mg. The discarded fractions precipitated by one-third saturation, and by more than two-thirds saturation, with ammonium sulphate contained 0.02 or less lecithinase units per mg.

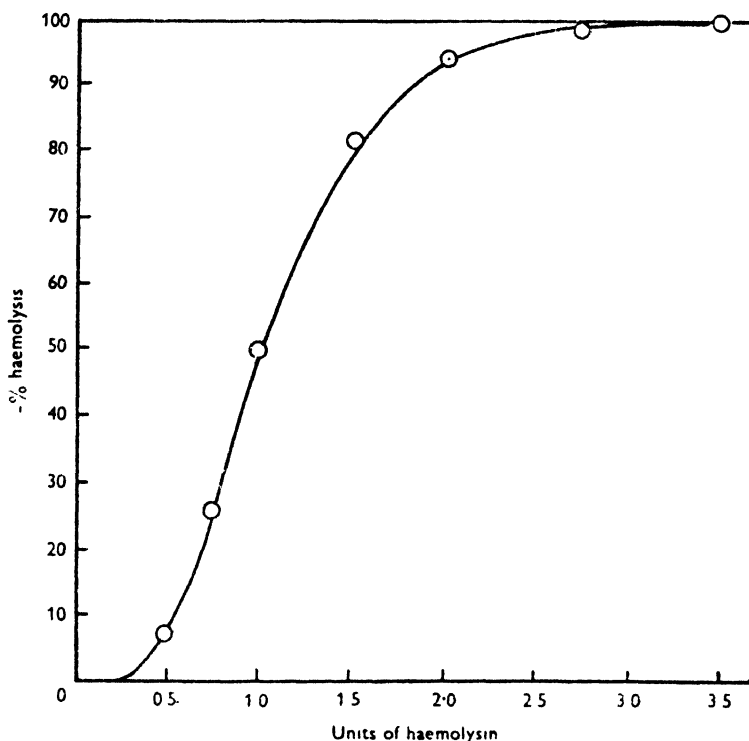


Fig. 2. Standard curve for titration of *B. cereus* haemolysin.

#### *Mode of hydrolysis of lecithin by Bacillus cereus lecithinase*

The mode of hydrolysis of lecithin by *B. cereus* lecithinase was compared with that of *Cl. welchii* lecithinase. Of three samples of 760 mg. of lecithin containing 30 mg. of P, one was hydrolysed by *B. cereus* lecithinase, one by *Cl. welchii* lecithinase and the third served as a control. The reaction mixtures were made up with distilled water to a total volume of 20 ml. and contained 0.005 M-CaCl<sub>2</sub>. On incubation the enzyme mixtures rapidly became acid and were frequently neutralized with 0.1 N-NaOH during the course of the hydrolysis. After 8 hr. at 37° the enzyme mixtures became fairly clear with a layer of fat on the surface, while the control still remained milky. The mixtures were then extracted four times with ether, and the ethereal solutions were separated by centrifugation. The combined ethereal solutions were evaporated to dryness, and extracted with acetone. The residues from the ether extracts of the

enzyme mixtures were almost completely soluble in acetone; that from the control mixture was completely insoluble, showing that most of the lecithin had been hydrolysed by the enzymes yielding acetone-soluble material. The acetone-soluble materials from both mixtures had the same acid value of 2.0, and thus appeared to be neutral fats.

Table 1. *Comparison of hydrolysis of lecithin by Clostridium welchii lecithinase and Bacillus cereus lecithinase*

|   | <i>B. cereus</i><br>lecithinase | <i>Cl. welchii</i><br>lecithinase | Control<br>(no enzyme) |
|---|---------------------------------|-----------------------------------|------------------------|
| Before hydrolysis:  |                                 |                                   |                        |
| Lecithin (mg.)  | 760                             | 760                               | 760                    |
| Total P (mg.)   | 29.9                            | 29.9                              | 29.9                   |
| Total N (mg.)   | 15.4                            | 15.4                              | 15.4                   |
| After hydrolysis:   |                                 |                                   |                        |
| (1) Water soluble products:                                 |                                 |                                   |                        |
| Total P (mg.)   | 29.0                            | 28.5                              | 0.3                    |
| Total N (mg.)   | 14.5                            | 14.0                              | 0.15                   |
| Inorganic P (mg.)   | 0                               | 0                                 | 0                      |
| Free choline (mg.)  | 0                               | 0                                 | 0                      |
| (2) Ether soluble products:                                 |                                 |                                   |                        |
| Acetone soluble material<br>(neutral fat) (mg.)             | 529.6                           | 510.0                             | 0                      |
| Acetone insoluble material<br>(unhydrolysed lecithin) (mg.) | 20.0                            | 31.0                              | 720                    |

Table 2. *The action of Bacillus cereus and Clostridium welchii lecithinases on free and protein-bound phospholipins*

| Substrate  | Time of<br>incubation | Acid soluble P (mg.) liberated<br>by the action of   |   |
|--|-----------------------|--|---|
|  |                       | 1 mg. <i>B. cereus</i><br>lecithinase<br>preparation | 1,900 ml.<br><i>Cl. welchii</i><br>lecithinase<br>preparation |
| 25 mg. of free lecithin  | { 15 min.<br>3 hr.    | 0.30<br>0.64   | 0.30<br>0.62  |
| 25 mg. of free phospholipins<br>extracted from lipovitellenin                  | { 15 min.<br>3 hr.    | 0.31<br>0.65   | 0.30<br>0.61  |
| 70 mg. of lipovitellenin con-<br>taining about 25 mg. of bound<br>phospholipin | { 15 min.<br>3 hr.    | 0.15<br>0.34   | 0.03<br>0.05  |

The aqueous solution from the two enzyme mixtures contained more than 95 % of the original lecithin P in an organic form and an equivalent amount of N but no free choline. In the case of *Cl. welchii* the organic P compound was crystallized as Ca salt and identified as phosphorylcholine (Macfarlane & Knight, 1941). By means of their technique 85 mg. of crystals were obtained from *B. cereus* lecithinase reaction mixture and 105 mg. from that of *Cl. welchii* lecithinase. Analysis showed that their compositions were very similar and agreed fairly well with that required for crystalline Ca compound of phosphorylcholine. (Ca, 12.0 and 12.0 %; N, 4.4 and 4.3 %; total P, 8.8 and 9.0 %;

free choline and inorganic phosphorus 0; first figures for the crystalline compound from *B. cereus* lecithinase reaction mixture and second figures for that from *Cl. welchii* lecithinase mixture.) Both crystalline organic P compounds were rapidly hydrolysed by a bone phosphatase preparation free from diesterase (kindly supplied by Dr M. G. Macfarlane). Equivalent amounts of free choline and inorganic phosphate were liberated from the two crystalline compounds after the hydrolysis, i.e. both compounds were phosphorylcholine. The changes of the three reaction mixtures after incubation are shown in Table 3. It can be seen that *B. cereus* lecithinase acts on lecithin in the same way as *Cl. welchii* lecithinase, in that neutral fat and phosphorylcholine are produced.

*Action of Bacillus cereus lecithinase on egg-yolk extract*

When egg-yolk extract was incubated with the enzyme preparation, there was a gradual increase of turbidity followed by formation of scum. Analysis of the reaction mixture showed a great increase of acid-soluble P as a result of the action of the lecithinase on the egg-yolk phospholipins. The close correlation of the lecithinase activity with the development of opalescence in egg-yolk extract was shown by the following experiment.

To a mixture of 10 ml. of diluted enzyme, 10 ml. of borate buffer (pH 7.1) and 20 ml. of distilled water at 37°, 20 ml. of yolk saline at the same temperature were added. Immediately and at intervals after mixing, the turbidities of 6 ml. samples were determined. As soon as each turbidity was read, 1 ml. 20% trichloroacetic acid was added to stop the reaction and the total acid-soluble phosphate estimated. The rate of liberation of acid-soluble P and the development of turbidity were almost parallel. There is little doubt that the production of turbidity in egg-yolk extract in this case is due primarily to the lecithinase activity, but the quantitative development of the egg-yolk reaction is determined also by a number of conditions which must be well controlled when the egg-yolk reaction is used for the titration of lecithinase.

*The action of Bacillus cereus and Clostridium welchii  
lecithinases on egg-yolk lipoproteins*

Under the above experimental conditions *B. cereus* lecithinase preparation was more active in producing turbidity in egg-yolk extract than *Cl. welchii* lecithinase. About 3 units of the latter is required to develop in egg-yolk extract a turbidity comparable to that produced by 1 unit of the former. As the egg-yolk reaction, also known as the lecitho-vitellin (L.V.) reaction, is supposed to be due to the action of lecithinase on egg-yolk lipoproteins, it was considered interesting to see if these two lecithinases also vary in their activities against purified egg-yolk lipoproteins. Lipovitellenin, the egg-yolk lipoprotein containing more phospholipins and with less phosphorus in its protein part, was chosen for this purpose. The preparation used contained 1.5% P, 9.9% N, and 4.99% choline. It could be dissociated with ethanol and repeatedly extracted with ether and ethanol to give 63% vitellenin (N, 15.4%; P, 0.23%; choline, nil) and 36% phospholipin (N, 1.8%; P, 3.54%; choline, 11.1%);

choline/P=0.79/1). From the analysis it can be seen that about four-fifths of the phospholipin was choline-containing and that any cephalin present could not be more than one-fifth of the total phospholipin. As shown in Table 2, similar amounts of *B. cereus* and *Cl. welchii* lecithinases, based on their rate of hydrolysis of free lecithin, also had about the same activity on the free phospholipin extracted from lipovitellenin, but varied very much in their activities against phospholipin bound in lipovitellenin. *Cl. welchii* lecithinase appeared to be much less active against vitellenin-bound lecithin as compared with *B. cereus* lecithinase. This might also account partially for its weaker

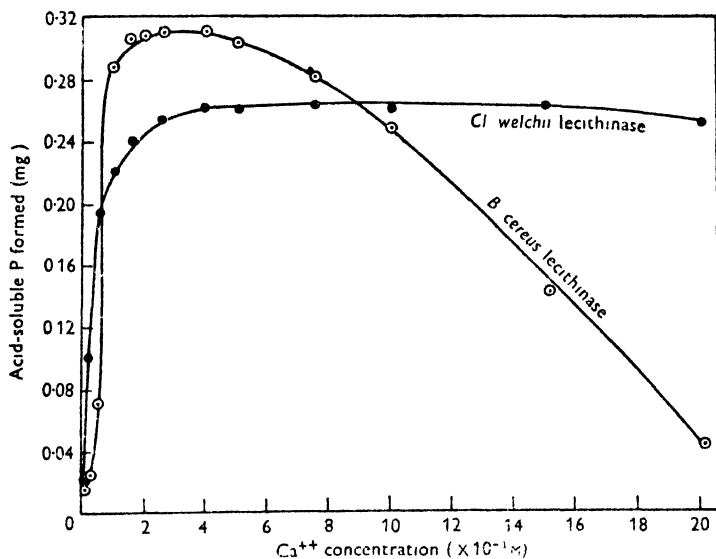


Fig. 3. Effect of Ca concentration on hydrolysis of free lecithin by *B. cereus* and *Cl. welchii* lecithinases. Reaction mixture: 1 mg. *B. cereus* lecithinase preparation or 0.001 ml. *Cl. welchii*  $\alpha$ -toxin; 1 ml. borate buffer pH 7.1; 1 ml. 2.5% lecithin; 0.0-0.8 ml. 0.1 M-CaCl<sub>2</sub>; water to 4 ml.; reaction time 15 min. at 37°.

activity on egg-yolk extract. As *B. cereus* lecithinase preparation also acts on cephalin, its relatively higher activity on lipovitellenin, as measured by the amount of acid-soluble P liberated, might be due to the additional effect of cephalinase. But estimation of the choline content of the hydrolysis products showed that over 90% of the acid-soluble P was, in fact, liberated from lecithin.

#### Characteristics of *Bacillus cereus* lecithinase

*The effect of Ca and Na ions on the lecithinase activity.* Like *Cl. welchii* lecithinase, the activity of *B. cereus* lecithinase is greatly affected by Ca ion. Fig. 3 compares the effects of Ca ion on *B. cereus* and *Cl. welchii* lecithinases. The latter has a wide range of optimal Ca concentration, whereas the activity of the former is maximal in the narrow range of  $1-4 \times 10^{-3}$  M-Ca. As lecithin is readily precipitated from an emulsion by Ca salts, it was first thought that the inhibitory effect of Ca ion at higher concentrations might be simply due

to precipitation of lecithin. In order to elucidate this, the effect of Ca ion on the lecithin emulsion itself was investigated. It was expected that lecithin emulsion would be stable at the concentration of Ca ion optimal for the lecithinase activity and precipitation might take place at a higher concentration. Contrary to these expectations, lecithin emulsion was found to be most unstable in the presence of  $1-4 \times 10^{-3}$  M-Ca, which is the optimal concentration for the activity of *B. cereus* lecithinase. This correlation is shown in Table 3.

Table 3. *The effect of calcium on the flocculation of lecithin and the activity of Bacillus cereus lecithinase*

|   | Concentration of Ca ( $10^{-3}$ M) |       |       |       |       |       |       |       |
|---|------------------------------------|-------|-------|-------|-------|-------|-------|-------|
|   | 25                                 | 10    | 5     | 2.5   | 1.25  | 0.5   | 0.25  | 0     |
| Rate of flocculation* of lecithin       | —                                  | —     | ++    | +++   | ++++  | +     | —     | —     |
| Lecithinase activity† (mg. P liberated) | 0.030                              | 0.230 | 0.300 | 0.312 | 0.310 | 0.075 | 0.024 | 0.022 |

\* The rate of flocculation of lecithin was roughly estimated by the relative time required for flocculation of lecithin after adding various amounts of  $\text{Ca}^{++}$  into lecithin emulsions. + + + +, + + +, + +, + = relative rates of flocculation; — = no flocculation after 3 hr.

† The lecithinase activity was expressed as mg. of acid-soluble phosphate liberated from 25 mg. of lecithin in 15 min. by the action of 1 mg. *B. cereus* lecithinase.

The fact that *B. cereus* lecithinase activity is greatest at the Ca ion concentration at which lecithin flocculates most readily from its emulsion suggests that the enzyme might have a maximal affinity for lecithin when the latter has absorbed an optimal amount of Ca ion in reaching its isoelectric point.

Although the hydrolysis of free phospholipins by *B. cereus* lecithinase requires the presence of free Ca ion, the egg-yolk reaction and the hydrolysis of phospholipins bound in lipovitellenin were found to proceed with maximal velocity even in the presence of oxalate. The addition of Ca ion, in concentration as low as  $10^{-3}$  M, greatly decreased these activities (see Fig. 4). The independence of *B. cereus* lecithinase of free Ca ion for the hydrolysis of phospholipin present in lipovitellenin might be due to a small amount of Ca (0.08 %) bound in the lipovitellenin which is not readily precipitated by oxalate and is sufficient to activate the enzyme. It is also possible that the combination of phospholipin with protein has so changed their configurations as to render Ca ion unnecessary for the action of the enzyme on the phospholipin.

Later, it was found that Na ion has a strong inhibitory action on the enzyme activity, the activity of the enzyme in physiological saline being only 5 % of that in water. Fig. 5 shows the amount of acid-soluble P liberated from lecithin by a given amount of *B. cereus* lecithinase at various concentrations of Na ion. A similar, but smaller effect was observed with *Cl. welchii* lecithinase.

*The effect of pH.* Since the enzyme activity is greatly affected by a small change in the Na concentration, and since sodium salt buffers of different pH generally vary in their Na content, it is necessary to equalize the final Na concentration of reaction mixtures containing buffers of different pH, otherwise the enzyme activity might be influenced not only by the difference

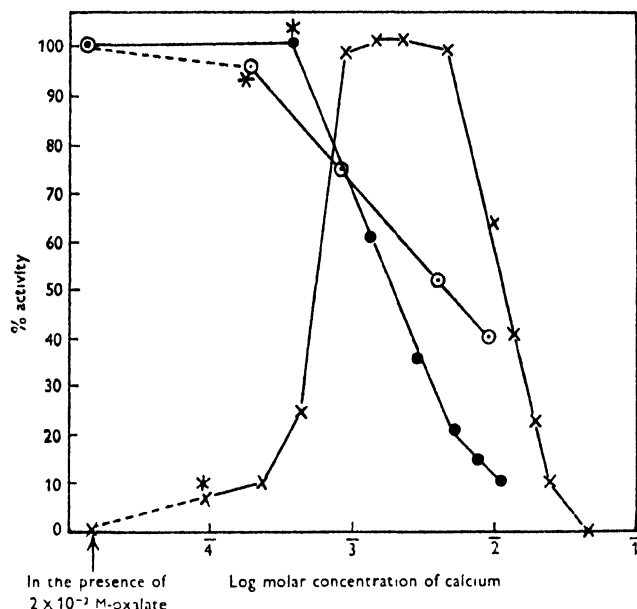


Fig. 4. The effect of Ca on hydrolysis of free and bound phospholipins by *B. cereus* lecithinase. x-x-x, hydrolysis of free lecithin; o-o-o, hydrolysis of phospholipins bound in lipovitellenin; ●-●-●, production of turbidity in egg-yolk extract. Percentage activity is calculated by assuming the maximal activity as 100%. (No Ca was added to these reaction mixtures; the small amount of Ca present came from the enzyme and the substrate.)

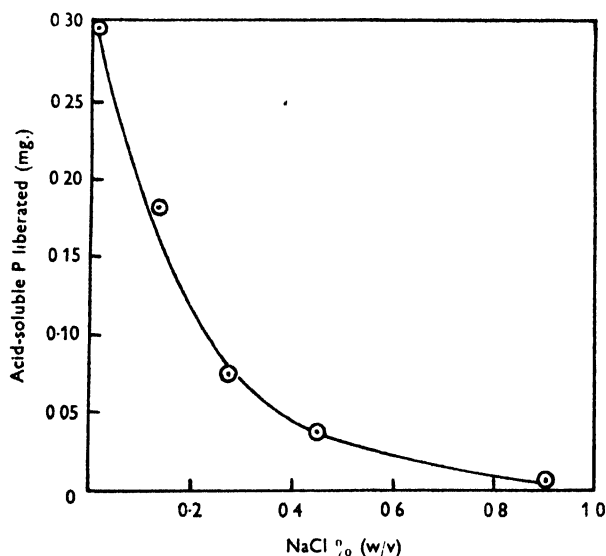


Fig. 5. The effect of Na concentration on lecithinase activity of *B. cereus*. Reaction mixture; 1 mg. *B. cereus* lecithinase preparation; 1 ml. borate buffer pH 7.1; 0.1 ml. 0.1 M-CaCl<sub>2</sub>; 1 ml. 2.5% lecithin; 0-0.6 ml. 6% (w/v) NaCl; water to 4 ml.; reaction time 15 min. at 37°.

of pH, but also by the variation of the salt concentration. Fig. 6 compares the enzyme activities at various pH's with and without adjustment of the final Na concentration. Borate buffers were used for pH 6.7-9.0 and acetate buffer for pH 5.7. The final strength of the buffer in the reaction mixture is 0.012 M. Although the optimal pH in both cases was around neutrality, the effect was greatly exaggerated in curve I, where the final salt concentrations were left unadjusted. This additional effect was doubtless due to the lower Na ion concentrations at pH's near neutrality.

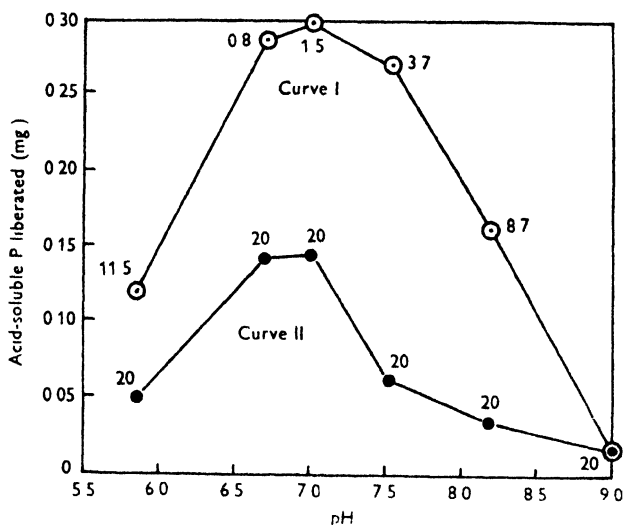


Fig. 6. The effect of pH on the activity of *B. cereus* lecithinase. Reaction mixture: 1 mg. *B. cereus* lecithinase preparation, 0.1 ml. 0.1 M-CaCl<sub>2</sub>; 1 ml. 2.5% lecithin; 1 ml. 0.05 M borate buffer pH 6.7-9.0, or acetate buffer pH 5.7; water to 4 ml.; reaction time 15 min. at 37°. Figures on the curves indicate the calculated [Na<sup>+</sup>] of the reaction mixtures (× 10<sup>-3</sup> M). The variation of [Na<sup>+</sup>] at various pH's as shown in curve I is due to the different Na content of the buffers. In curve II, the [Na<sup>+</sup>] of the reaction mixture at various pH's were all adjusted to that at pH 9, i.e. 20 × 10<sup>-3</sup> M by the addition of NaCl solution.

**Effect of substrate concentration.** Fig. 7 shows the effect of substrate concentration on the initial velocity of hydrolysis with a given concentration of enzyme. Since lecithin is present as a colloidal solution, it is difficult to calculate the Michaelis constant (*K<sub>m</sub>*) for the enzyme. Following the method of calculation, as used by Zamecnik, Brewster & Lipmann (1947) for *Cl. welchii* lecithinase, and making similar assumptions, the *K<sub>m</sub>* of *B. cereus* lecithinase is calculated to be 4.8-5.4 × 10<sup>-3</sup> M, which is near to that of *Cl. welchii* lecithinase, viz. 5 × 10<sup>-3</sup> M.

**Effect of enzyme concentration.** Fig. 8 shows that the initial velocity of hydrolysis, as measured by determining the amount of acid-soluble P formed in 15 min., is directly proportional to the enzyme concentration over at least an eightfold range.

**The stability of the enzyme.** Like *Cl. welchii* lecithinase, the *B. cereus* enzyme



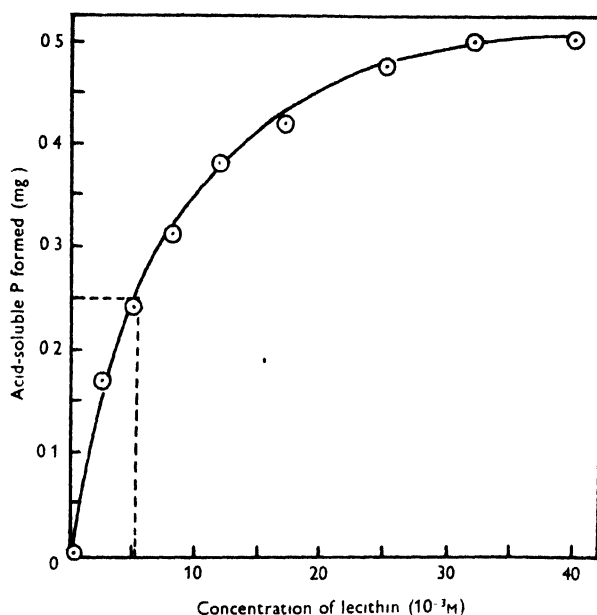


Fig. 7. The effect of substrate concentration. Enzyme concentration 0.25 mg./ml.; pH 7.1; total volume of reaction mixture 4 ml.; reaction time 15 min. at 37°.

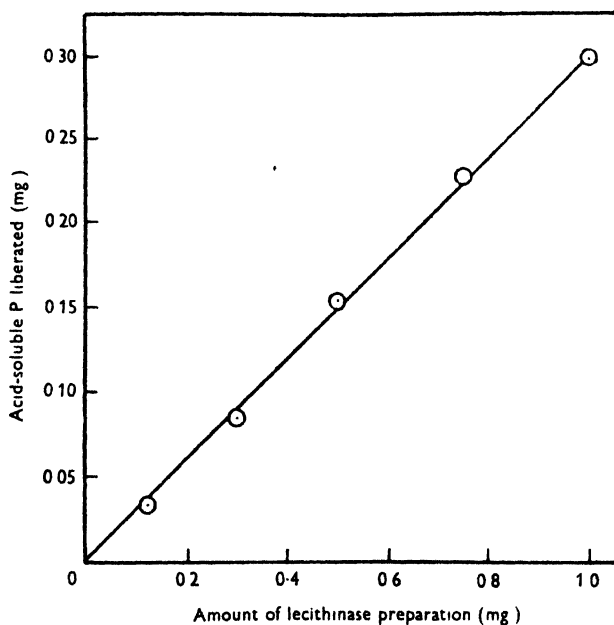


Fig. 8. The effect of enzyme concentration. Reaction mixture: 25 mg. lecithin; 1 ml. borate buffer pH 7.1; 0.1 ml. 0.1 M-CaCl<sub>2</sub>; enzyme preparation 0.1-1.0 mg.; water to 4 ml.; reaction time 15 min. at 37°.

is fairly heat stable. Heating the enzyme in pH 7.1 borate buffer for 30 min. at 60° in a sealed tube destroyed only 20 % of its activity, and 30 % of its activity still remained after standing in boiling water for 10 min. The enzyme is also resistant to oxidation; treatment with a 1:100 dilution of 30 vol.  $\text{H}_2\text{O}_2$  for 1 hr. at room temperature had no effect on its activity. It is rather easily inactivated by surface denaturation; bubbling  $\text{N}_2$  through a dilute enzyme solution for 60 min. destroyed 40 % of its activity. It can also be inactivated by incubating with dilute formalin. When kept in 50 % aqueous glycerol at 2°, the enzyme activity remained unchanged for over 2 years. Dilute solutions of the enzyme in water or saline are rather unstable at room temperature.

*The haemolytic activity of Bacillus cereus lecithinase preparations*

The close association of lecithinase activity and haemolytic activity of *B. cereus* and *B. mycoides* has already been discussed (McGaughey & Chu, 1948). One lecithinase unit is generally associated with ten haemolytic units. Further studies showed that this haemolysin has many characteristics in common with *Cl. welchii* lecithinase, which is also haemolytic.

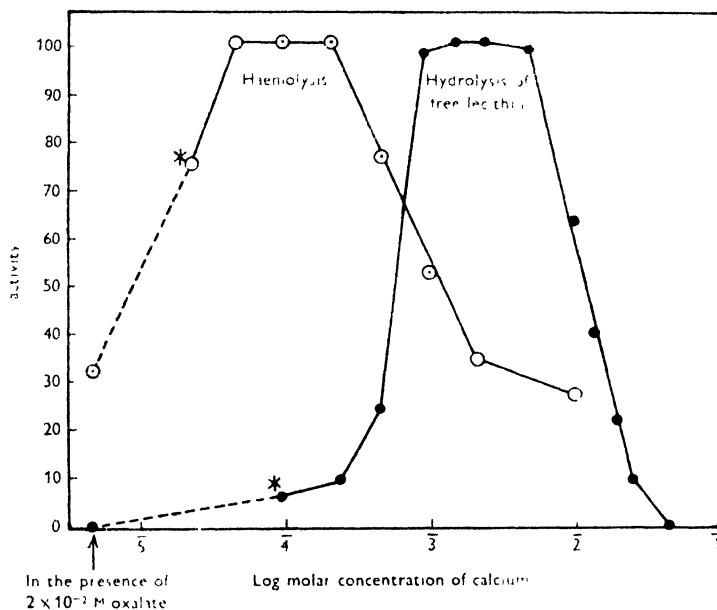


Fig. 9. The effect of Ca on haemolytic and lecithinase activities of *B. cereus*. No Ca was added to these reaction mixtures, the small amount of Ca present as shown in the figure came from the enzyme and the substrate.

**Effect of Ca ions.** The lecithinase activity of *B. cereus* is optimal at Ca concentration around  $1-4 \times 10^{-3}$  M. The haemolytic activity is maximal at a lower concentration of Ca, ranging between  $0.05-0.2 \times 10^{-3}$  M. The activity can be decreased either by very slight increase of Ca concentration or by the addition of citrate or oxalate as shown in Fig. 9.

*Susceptibility of erythrocytes of different species.* Oakley & Warrack (1941) showed that  $\alpha$ -toxin of *Cl. welchii* was comparatively inactive against goat or horse red blood cells; *B. cereus* haemolysin behaves in the same way. Red cells of sheep, pig, rabbit, guinea-pig and man are almost equally susceptible to its action; horse cells less susceptible; and goat cells very resistant. About twenty times more haemolysin is required to haemolyse goat cells than sheep cells.

Table 4. *The inhibition of lecithinase activity and haemolytic activity of Bacillus cereus by normal horse serum*

Lecithinase activity: 0.0-0.2 ml. normal horse serum diluted with saline to 0.2 ml. were allowed to act with 1 ml. of diluted enzyme in water at 37° for 15 min., one ml. pH 7.1 borate buffer, 0.1 ml. 0.1 M-CaCl<sub>2</sub>, 0.7 ml. H<sub>2</sub>O and 1 ml. 2.5 % lecithin were then added and the amount of hydrolysis estimated in the usual way after another 15 min. incubation.

Haemolytic activity. Decreasing amounts of normal horse serum were incubated at 37° for 15 min. with 0.1 mg. of lecithinase preparation containing 3 H.U. in a total volume of 2 ml. made up with borax buffer saline. 1 ml. standard suspension of red cells were then added and the degree of haemolysis determined after 1 hr. incubation.

| Amount of normal horse serum (ml.) | Lecithinase activity of 1 mg. lecithinase preparation |                | Haemolytic activity of 0.1 mg. lecithinase preparation |                |
|------------------------------------|---|----------------|--|----------------|
|                                    | P liberated (mg.)                                     | Inhibition (%) | Haemolysis (%)   | Inhibition (%) |
| 1/5                                | 0.000   | 100.0          | 0  | 100            |
| 1/10                               | 0.001   | 99.6           | 0  | 100            |
| 1/20                               | 0.004   | 98.5           | 0  | 100            |
| 1/40                               | 0.028   | 90.0           | 0  | 100            |
| 1/80                               | 0.050   | 82.5           | 0  | 100            |
| 1/160                              | 0.110   | 62.3           | 0  | 100            |
| 1/320                              | 0.156   | 45.0           | 0  | 100            |
| 1/640                              | 0.222   | 25.4           | 0  | 100            |
| 1/1280                             | 0.256   | 9.9            | 3.0  | 97             |
| 1/2560                             | —   | —              | 50.0   | 50             |
| 1/5120                             | —   | —              | 97.0   | 3              |
| 0                                  | 0.284   | —              | 100.0  | —              |

*Thermostability.* The effect of heating on *B. cereus* haemolysin is interesting. The haemolytic activity was completely destroyed after heating at 60° for 10 min., while boiling for 10 min. only destroyed 80 % of its activity. This peculiar heat resistance has also been observed with staphylococcus  $\alpha$ -haemolysin (Arrhenius, 1907) and *Cl. welchii*  $\alpha$ -toxin. (Guillaumie, Kreguer & Fabre, 1946). Heating affects the haemolytic activity of *B. cereus* filtrate more than it affects the lecithinase activity. Heating at 60° for 10 min. has very little effect on lecithinase activity but completely abolishes the associated haemolytic activity. However, this does not necessarily mean that the haemolysin is unrelated to the lecithinase, as it is quite possible that heating at 60° only upsets the access of lecithinase to the substrate on the red cells but does not affect the approach of the enzyme to free lecithin. The haemolytic activity of *Cl. welchii*  $\alpha$ -toxin is also more sensitive to heat than its associated lecithinase activity.

*Hot-cold effect.* Unlike *Cl. welchii*  $\alpha$ -toxin (van Heyningen, 1941 b), *B. cereus* haemolysin is not a hot-cold lysin.

**Resistance to oxidation.** Like *Cl. welchii*  $\alpha$ -toxin, the haemolytic activity of *B. cereus* is not affected by oxidation.

**Inhibition by normal serum.** Lysis of red cells as well as hydrolysis of lecithin by *B. cereus* are both strongly inhibited by normal sera of all the animals tested, including horse, sheep, rabbit, ox, guinea-pig, mouse and man (see below).

**Effect on peptone.** The activity of *B. cereus* haemolysin is greatly enhanced in the presence of a small amount of peptone. About 50 % more haemolysin is required to lyse sheep red cells in the absence of peptone than in its presence. Similar observation has been made on *Cl. welchii* toxin by Oakley & Warrack (1941).

#### *Inhibition of lecithinase activity by normal serum*

The hydrolysis of free lecithin and the lysis of red blood cells by *B. cereus* filtrate were strongly inhibited by normal sera of all the animals tested, including horse, ox, sheep, rabbit and man. The inhibitor was found to be associated with both serum globulin and serum albumin. But it was not

Table 5. *The effect of normal serum proteins on the hydrolysis of free phospholipins and protein-bound phospholipins by Bacillus cereus phospholipinase*

Reaction mixture: 1 mg. *B. cereus* lecithinase; 1 ml. borate buffer; 0.1 ml. 0.1 M-CaCl<sub>2</sub> (only for the hydrolysis of free phospholipins); 1 ml. of 2.5 % free phospholipins or 7 % lipovitellenin; serum proteins as indicated above; water to 4 ml. Reaction time: 15 min. at 37°.

|                              | Hydrolysis of free phospholipin   |                | Hydrolysis of bound phospholipins  |                |
|------------------------------|---|----------------|--|----------------|
|                              | 'P' liberated from 25 mg. free phospholipins extracted from 70 mg. lipovitellenin | Inhibition (%) | 'P' liberated from 70 mg. of lipovitellenin containing about 25 mg. bound phospholipin | Inhibition (%) |
| Control. No serum protein    | 0.307   | —              | 0.140  | —              |
| Horse serum globulin 0.2 mg. | 0.090   | 71.0           | 0.141  | 0              |
| Horse serum globulin 1.0 mg. | 0.061   | 80.0           | 0.142  | 0              |
| Horse serum albumin 0.2 mg.  | 0.060   | 80.5           | 0.141  | 0              |
| Horse serum albumin 1.0 mg.  | 0.045   | 85.5           | 0.143  | 0              |

destroyed by heating to 70° for 10 min., which had apparently denatured the serum proteins. The extent of the inhibition is shown in Table 4. In the experiments cited the inhibition was measured by comparing the rate of hydrolysis during the first 15 min. of incubation. The inhibitory effect has also been demonstrated in experiments where the reaction was allowed to proceed for several hours. The inhibition is thus not just a temporary lag effect.

It is interesting that normal serum proteins, although inhibiting the hydrolysis of free lecithin in high dilution, have very little effect on the yolk reaction. This is partially explained by the finding that normal serum proteins also fail to inhibit the hydrolysis of phospholipins bound in lipovitellenin and lipovitellin, the two egg-yolk lipoproteins (Table 5). The fact that *B. cereus*

lecithinase preparation also acts on cephalin and that the cephalinase activity is not inhibited by normal serum, suggested that the acid-soluble P liberated from lipovitellenin in the presence of normal serum proteins might largely come from cephalin. This possibility was ruled out, however, by the finding that the choline contents of the acid-soluble P compounds liberated from lipovitellenin in the presence of normal serum proteins or in its absence were practically the same. The reason why the combination of lecithin with proteins like vitellenin or vitellin should counteract the inhibition of the lecithinase by serum protein is not clear.

*Production of specific anti-lecithinase in rabbits and its differentiation from normal serum inhibitor*

Specific *B. cereus* anti-lecithinase has been produced by immunizing rabbits with the enzyme preparation. Unlike the inhibitor in normal serum the specific anti-lecithinase is capable of inhibiting not only the hydrolysis of free lecithin but also the yolk reaction and the hydrolysis of protein-bound lecithin. Possibly, the specific anti-lecithinase has a more direct effect on the enzyme than the inhibitor present in normal serum. Table 6 compares the effect of normal rabbit serum and immune rabbit serum on various activities on *B. cereus* lecithinase preparation.

Table 6. *The effect of normal rabbit serum and immune rabbit serum on various activities of Bacillus cereus lecithinase preparation*

This table compares the effects of 0.1 ml. normal serum and 0.1 ml. immune serum on the various activities produced by 1 mg. of *B. cereus* lecithinase preparation.

|                      | Lecithinase activity        |                |   |                |
|----------------------|-----------------------------|----------------|---|----------------|
|                      | Hydrolysis of free lecithin |                | Hydrolysis of phospholipins bound in lipovitellenin |                |
|                      | P liberated (mg.)           | Inhibition (%) | P liberated (mg.)                                   | Inhibition (%) |
| Control (no serum)   | 0.310                       | —              | 0.140   | —              |
| Normal serum 0.1 ml. | 0.005                       | 98             | 0.140   | 0              |
| Immune serum 0.1 ml. | 0.005                       | 98             | 0.055   | 61             |

|                      | Haemolytic activity |                | Yolk reaction         |                | Toxicity               |                |
|----------------------|---------------------|----------------|-----------------------|----------------|------------------------|----------------|
|                      | Haemolysis (%)      | Inhibition (%) | Increase of turbidity | Inhibition (%) | mice dying mice tested | Protection (%) |
| Control (no serum)   | 100                 | —              | 168*                  | —              | 12/12                  | —              |
| Normal serum 0.1 ml. | 0                   | 100            | 167                   | 0              | 12/12                  | 0              |
| Immune serum 0.1 ml. | 0                   | 100            | 8                     | 95             | 3/12                   | 75             |

\* Turbidity expressed as mg. BaSO<sub>4</sub>/100 ml.

Antisera against *B. cereus* lecithinase have no effect on *Cl. welchii* lecithinase, and vice versa; the enzymes therefore are antigenically different. On the other hand, lecithinase produced by *B. mycoides* can be neutralized by antisera against *B. cereus* lecithinase. This provides further evidence of their close relationship.

*The toxicity of Bacillus cereus lecithinase*

The fact that the hydrolysis of free lecithin and lysis of red blood cells by *B. cereus* lecithinase are inhibited by normal serum suggests that the enzyme might be innocuous *in vivo*. On the other hand the inability of normal serum to inhibit the hydrolysis of lecithin bound in egg-yolk lipoprotein suggests that normal serum might fail to inhibit the action of the enzyme on other lipoproteins occurring in animal tissue; the enzyme might therefore not be so innocuous *in vivo* as first expected. In fact, the enzyme preparation was found to be toxic for experimental animals, although the toxicity might be due to factors other than lecithinase.

Intravenous inoculation of a dose of the enzyme preparation containing 2-4 lecithinase units is lethal to mice. The M.L.D. of *Cl. welchii*  $\alpha$ -toxin for mice, on the other hand, generally contains 0.5 lecithinase unit. Intradermal injection in rabbits and guinea-pigs produces marked inflammatory lesions with central necrosis. The toxicity can be neutralized by immune rabbit sera containing specific anti-lecithinase. As the enzyme preparation is very crude, it is impossible to say whether its toxicity is due to lecithinase activity exclusively or to some other factors as well. The enzyme preparation has already been found to possess cephalinase activity which is likely to account for part of its toxicity.

*The action of Bacillus cereus lecithinase preparation on cephalin*

Unlike the *Cl. welchii* lecithinase (Macfarlane, 1942) the preparation from *B. cereus* was capable of hydrolysing not only lecithin but also cephalin. Whether the hydrolysis of cephalin is due to the lecithinase or to another enzyme remains to be seen. A few preliminary experiments have shown that acid-soluble organic P but not inorganic P is split from cephalin by the enzyme. An enzyme preparation containing three lecithinase units was able to catalyse the production of 0.12 mg. acid-soluble P from 25 mg. of cephalin in 15 min. under the same conditions as used for lecithinase estimation. The cephalinase activity also requires the presence of Ca ion, but is not inhibited by normal serum, which has a very strong inhibitory effect on the lecithinase activity.

## DISCUSSION

The data showing the close similarity of *B. cereus* and *Cl. welchii* lecithinases in their enzymic properties and biological activities are summarized in Table 7. *B. cereus* lecithinase possesses most of the biological activities found to be associated with *Cl. welchii*  $\alpha$ -toxin. Although these two lecithinases are very similar in most respects, differences have been noted between them. Some of these differences may have a bearing on their activities *in vivo*; the different reactions to normal serum is an interesting example. Thus, although these two enzymes exhibit similar chemical activities *in vitro*, they might differ very much in their activities *in vivo*.

The inhibitory action of normal serum protein on the hydrolysis of free

lecithin by *B. cereus* lecithinase, in contrast to its failure to suppress the hydrolysis of lecithin bound in egg-yolk lipoproteins is another illustration of the complexity of the biological system.

Table 7. *Comparison of Bacillus cereus lecithinase and Clostridium welchii lecithinase*

| Lecithinase activity | Ca requirement<br>Optimal pH<br>Thermostability (%):                        | <i>B. cereus</i><br>lecithinase | <i>Cl. welchii</i><br>lecithinase |
|----------------------|---|---------------------------------|-----------------------------------|
|                      |   | +                               | +                                 |
|                      |   | 6.8-7.2                         | 7.0-7.6                           |
|                      | activity after { 60° 10 min.<br>boiling 10 min.                             | 82<br>32                        | 87<br>45                          |
|                      | Resistance to oxidation   | +                               | +                                 |
|                      | Inactivation by formalin  | +                               | +                                 |
|                      | Precipitation by 2/3 sat'd. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | +                               | +                                 |
|                      | Activity on egg-yolk lipoproteins   | + +                             | +                                 |
|                      | Inhibition by normal serum proteins   | +                               | -                                 |
| Biological activity  | Egg-yolk reaction and Nagler reaction                                       | +                               | +                                 |
|                      | Haemolytic activity   | +                               | +                                 |
|                      | Inhibition of haemolysis by normal serum proteins                           | +                               | -                                 |
|                      | Dermonecrotic effect in guinea-pigs   | +                               | +                                 |
|                      | Toxicity to mice  | +                               | ++                                |

I wish to express my gratitude to Prof. C. A. McGaughey and Prof. W. I. B. Beveridge for their interest and encouragement in this work; and to Dr W. E. van Heyningen and Dr Dorothy Needham for their advice and help and for reading the manuscript. My thanks are also due to Dr M. G. Macfarlane for her supply of the monophosphatase.

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## The Incidence of Penicillin-sensitive Variant Colonies in Penicillinase-producing Strains of *Staphylococcus pyogenes*

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**SUMMARY:** Penicillinase-producing strains of *Staphylococcus pyogenes* isolated from human infections and kept under various conditions were plated out and fifty colonies from each plate tested for penicillin-sensitivity to determine the permanence of their resistance to penicillin.

Of 32 strains kept in Lemco broth and tested once, 5-12 months after isolation, two yielded only penicillin-sensitive colonies and fifteen others yielded a proportion of such.

Of six strains preserved by the gelatin-ascorbic acid drying process and kept for over a year, only one yielded penicillin-sensitive colonies.

Of six strains kept in Lemco broth with and without regular subculture and tested at intervals for 9 months, all gave rise to at least one penicillin-sensitive variant colony and more than half the colonies of one strain became penicillin-sensitive.

The natural tendency of these strains to yield penicillin-sensitive variant colonies was not appreciably accelerated by treatment with X-rays or by growing them with other organisms.

From many studies on penicillin-resistant staphylococci it is clear that the cocci display two fundamentally different types of resistance; this was first pointed out by Spink, Hall & Ferris (1945) and has been confirmed by many workers since. First, there is the type of resistance acquired *in vitro* by serial subculture of staphylococci in increasing concentrations of penicillin. The organisms often show gross changes in morphology, cultural characteristics and virulence, and tend to revert to the typical penicillin-sensitive variety after repeated subculture in the absence of penicillin. The resistance is not usually associated with the production of penicillinase. Bellamy & Klimek (1948), however, describe a strain trained *in vitro* to grow in penicillin '4 mg./ml.', which produced small amounts of penicillinase, but only when grown in the presence of penicillin. This strain had lost many of the properties of a staphylococcus. It consisted of Gram-negative cocci and rods and had lost the ability to ferment lactose, sucrose, maltose, mannitol and galactose. The second type of resistance occurs in strains isolated from infective processes and human carriers; these strains do not differ from typical penicillin-sensitive strains of *Staphylococcus pyogenes* in morphology, cultural characteristics or virulence. As first pointed out by Kirby (1944), this type of resistance is characterized by the production of a powerful penicillin-inactivator. In reports by Spink *et al.* (1945) Bondi & Dietz (1945), Gots (1945), Barber (1947*a*, *b*) and Barber & Rozwadowska-Dowzenko (1948), all penicillin-resistant strains of *Staph. pyogenes* isolated *in vivo* were shown to destroy penicillin.

During the past two years penicillin-resistant strains of *Staph. pyogenes*

were isolated at Hammersmith Hospital from 111 patients with staphylococcal infection, and a number were obtained from nasal and skin carriers (Barber, 1947 *a, b*; Barber & Rozwadowska-Dowzenko, 1948). All these strains were able to destroy penicillin. Single colonies of penicillin-resistant cocci appearing on the original culture plates of human material were subcultured into Lemco broth and maintained in the laboratory, many of them for from 9 to 18 months. In the present investigation forty-four of these cultures were studied to determine the permanence or impermanence of their resistance to penicillin.

## METHODS

*Penicillin ditch-plates.* Ditches were cut at one side of Lemco agar plates and filled with the same medium containing approximately 10 units penicillin/ml. The ditch-plates were prepared 24 hr. or more before use to allow some penicillin to diffuse out of the ditch. As a control, the Oxford staphylococcus was streaked across every ditch-plate at the time of its use.

*Penicillinase production* was tested for by the method of Barber (1947 *a*).

*Typing by bacteriophage.* Parent and variant cultures were phage-typed by Dr Allison by the method of Wilson & Atkinson (1945).

## RESULTS

*Variants from thirty-two strains kept in Lemco broth for 5-12 months.* Thirty-two strains kept, with occasional subculture, in Lemco broth were plated out on Lemco agar plates and from each plate fifty colonies were picked off and streaked across penicillin ditch-plates. The results are summarized in Table 1. Of the 32 strains tested seventeen gave penicillin-sensitive colonies and from

Table 1. *Distribution of percentage of colonies of penicillin-sensitive Staphylococcus pyogenes in thirty-two penicillin-resistant strains (fifty colonies of each strain tested.)*

| No. of strains | Percentage |
|----------------|------------|
| 2              | 100        |
| 5              | > 50       |
| 3              | 22-50      |
| 3              | 10-20      |
| 1              | 4          |
| 3              | 2          |
| 15             | 0          |

two cultures all fifty colonies tested were penicillin-sensitive. All the penicillin-sensitive colonies appeared to be as sensitive as the Oxford staphylococcus and all the resistant colonies grew across the ditch (Pl. 1, fig. 1). No intermediate degrees of resistance were encountered. The penicillin-sensitive colonies bred true on subculture and proved to have lost their capacity to destroy penicillin.

The parent broth cultures were then plated directly on to penicillin ditch-plates. The two cultures from which only penicillin-sensitive colonies were obtained (Pl. 1, fig. 2) showed no penicillin-resistant colonies even by this method, which can detect as few as one in 10,000 resistant colonies (see Barber,

1947*b*). The fifteen cultures yielding a mixture all resembled wholly penicillin-resistant strains and no colonial variation was detectable (Pl. 1, fig. 3). This is presumably because a few resistant organisms produce penicillinase sufficient to permit growth of the penicillin-sensitive organisms. In artificial mixtures of penicillin-sensitive and penicillin-resistant organisms the ratio of sensitive to resistant had to be at least 100:1 if the sensitive organisms were to be detected on a penicillin ditch-plate (Pl. 1, fig. 4). One interesting point is that whereas penicillin-sensitive colonies get smaller as the penicillin is approached, the penicillin-resistant colonies get larger. This is particularly well seen in Pl. 1, fig. 4.

*Variants from six strains preserved by the gelatin-ascorbic acid drying process.* Six strains kept for over a year after drying by Stamp's (1947) method yielded far fewer variants. From five of the cultures no penicillin-sensitive colonies were isolated and one strain yielded only three.

Table 2. *Incidence of penicillin-sensitive variants in six strains kept under controlled conditions for 9 months*

|              | No. of penicillin-sensitive colonies out of fifty tested |          |          |          |          |          |          |          |          |          |            |          |
|--------------|--|----------|----------|----------|----------|----------|----------|----------|----------|----------|------------|----------|
|              | D3R  |          | D6R      |          | P193R    |          | P268R    |          | Sp249R   |          | P5155RR    |          |
| Tested after | <i>a</i>   | <i>b</i> | <i>a</i> | <i>b</i> | <i>a</i> | <i>b</i> | <i>a</i> | <i>b</i> | <i>a</i> | <i>b</i> | <i>a</i> * | <i>b</i> |
| 6 weeks      | 0  | 0        | 0        | 0        | 0        | 0        | 1        | 0        | 13       | 5        | 6          | 1        |
| 3 months     | 0  | 0        | 0        | 1        | 0        | 0        | 0        | 0        | 9        | 3        | 5          | 0        |
| 4 months     | 0  | 0        | 0        | 1        | 0        | 0        | 0        | 0        | 28       | 12       | 3          | 0        |
| 5 months     | 0  | 0        | 0        | 0        | 0        | 0        | 3        | 1        | 25       | 20       | 5          | 0        |
| 6 months     | 0  | 0        | 0        | 1        | 0        | 0        | 0        | 1        | 36       | 25       | 4          | 3        |
| 9 months     | 0  | 1        | 0        | —        | 1        | 0        | 0        | 0        | 34       | 40       | 41         | 6        |
| 12 months    | 0  | 0        | 0        | —        | 0        | 0        | 0        | —        | 42       | 31       | 50         | 11       |

*a* = Subcultured twice a week in Lemco broth.

*b* = Left on bench in Lemco broth and subcultured every 3 months.

— = Not tested.

\* This culture became contaminated with a bacillus between the fourth and fifth months.

*Variants from six strains kept under controlled conditions for 9 months.* Six cultures were studied more carefully over a period of 9 months; five of these had been isolated within a few weeks of starting the experiment and one, P5155RR, was a penicillin-resistant colony from a culture isolated 7 months previously and at that time yielding a mixture of sensitive and resistant colonies. One culture of each strain was subcultured twice a week in Lemco broth, and one was left on the bench in the same medium and subcultured once during the period. Each of the two series was plated out after 1½, 3, 4, 5, 6, 9 and 12 months; and, except for two strains left without subculture that had died at 9 and 12 months, fifty colonies from each strain were tested on the ditch-plates. In all 600–700 colonies were tested from each of the twelve cultures (Table 2). From all six strains penicillin-sensitive variant colonies were isolated, although two yielded only a single variant and two more only a few. Strain Sp249R yielded an increasing number of penicillin-sensitive

colonies and subculture seemed to increase the rate of conversion. For the first 6 months strain P5155RR, when subcultured, gave a few variant colonies at each test. At the ninth month, however, there were forty-one colonies and at the twelfth fifty. This specimen became contaminated with a bacillus between the fourth and fifth months. When left on the bench the strain yielded only one variant in the first 5 months and then at subsequent tests a steadily increasing proportion.

As in the previous experiment, nearly all the colonies tested were either as sensitive as the Oxford staphylococcus or grossly resistant, growing right across the penicillin ditch. Occasionally, however, colonies showed an intermediate degree of resistance; but when such streaks were emulsified in broth, plated on plain agar and fifty colonies again tested, a mixture of penicillin-sensitive and resistant organisms was found. As before, penicillin-sensitive colonies bred true and had lost the capacity to destroy penicillin.

#### *Attempts to stimulate variation*

Two agents were tested for power to accelerate the natural tendency of penicillinase-producing staphylococci to give rise to penicillin-sensitive variants.

*The effect of X-radiation.* A dose of 1,000,000 r. (about the maximum dose leaving any surviving staphylococci) was used on strains D3 and D6, which naturally produced only occasional penicillin-sensitive variants. The irradiated cultures were plated out after 1, 2, 6, 12, 20 and 26 subcultures and fifty colonies examined. Of the 300 colonies tested from each after X-raying, none was penicillin-sensitive.

*The effect of mixing cultures with Streptococcus pyogenes.* Following the work of Voureka (1948), *Streptococcus pyogenes* (Group A) was mixed with penicillin-resistant staphylococci. Three strains of *Strep. pyogenes*, including the strain Milne used by Voureka, and ten strains of staphylococci, some of which were producing many penicillin-sensitive variants and some only producing a few, were used. The streptococci were grown for 18–24 hr. in Hartley broth and the cultures then seeded with two drops of a broth culture of one of the staphylococci. The mixtures were incubated for 24 hr., plated and fifty staphylococcal colonies tested. Control cultures of Hartley broth, seeded with a staphylococcus only, were similarly treated. There was (Table 3) no obvious difference in the number of variants occurring in the mixed cultures and in the controls.

*The effect of mixing penicillin-resistant and penicillin-sensitive strains of Staph. pyogenes.* A penicillin-sensitive (S) strain and a penicillin-resistant (R) strain (D3) were mixed in the following proportions: S/R 1:1, 1:100, 100:1. The mixtures were divided into two parts, one subcultured twice a week in Lemco broth and the other left on the bench in the same medium. At varying intervals during 5 months the mixtures were tested (Table 4). The S/R 1:1 mixture subcultured twice a week yielded penicillin-sensitive colonies in proportions varying from 1:1 to 4:1, whereas the culture left on the bench gave an increasing number of penicillin-sensitive colonies until all fifty tested were sensitive. Even at this stage, however, when the culture was plated directly on

to a penicillin ditch-plate, a few resistant colonies were isolated. The S/R 1:100 mixture when subcultured became and remained a nearly equal mixture and when left on the bench yielded a few sensitive colonies for the first 4 months and suddenly at the fifth month became predominantly penicillin-sensitive. With

Table 3. *Incidence of penicillin-sensitive variants after mixing with Streptococcus pyogenes*

| Strain  | No. of penicillin-sensitive colonies out of fifty tested |      |       |                  |    |
|---------|--|------|-------|------------------|----|
|         | Mixed with <i>Strep. pyogenes</i> , strain               |      |       | Control cultures |    |
|         | Richards   | C203 | Milne | 1                | 2  |
| D3R     | 1  | 0    | 0     | 0                | 0  |
| D6R     | 0  | 0    | 0     | 0                | 0  |
| Sp240R  | 43   | 41   | 32    | 36               | 44 |
| P5155RR | 15   | 10   | 15    | 10               | 9  |
| P6395R  | 6  | 4    | —     | 8                | —  |
| B7007R  | 14   | 16   | —     | 18               | —  |
| P7004R  | 29   | 26   | —     | 21               | —  |
| P7108R  | 12   | 20   | —     | 33               | —  |
| P268R   | —  | —    | 0     | 0                | —  |
| P193R   | —  | —    | 0     | 1                | —  |

Table 4. *Incidence of penicillin-sensitive colonies in mixtures of penicillin-sensitive and penicillin-resistant strains of Staphylococcus pyogenes*

| Tested after  | No. of penicillin-sensitive colonies out of fifty tested |     |           |    |           |    |                          |
|---------------|--|-----|-----------|----|-----------|----|--------------------------|
|               | S/R 1:1  |     | S/R 1:100 |    | S/R 100:1 |    | S/R 1:3<br>X-irradiation |
|               | a  | b   | a         | b  | a         | b  |                          |
| (Immediately) | 19   | 34  | 4         | —  | 49        | —  | 40                       |
| 2 weeks       | —  | —   | 2         | 1  | 50        | 50 | 39                       |
| 4 or 6 weeks  | 29   | 47  | 23        | 3  | 44        | 50 | 16                       |
| 2 months      | 17   | 48  | 34        | 6  | 41        | 50 | 20                       |
| 3 months      | 10   | 49  | 26        | 3  | 49        | 39 | 34                       |
|               |  |     |           |    | 48        | 39 |                          |
| 4 months      | 14   | 50  | 20        | 7  | 35        | 14 | 48                       |
|               |  |     |           |    | 34        | 11 |                          |
| 5 months      | 16   | 50* | 21        | 42 | 50*       | 30 | 50                       |

a Subcultured twice a week in Lemco broth.

b = Left on bench in Lemco broth.

— = Not tested.

\* Penicillin-resistant colonies isolated when whole culture was plated on a ditch-plate.

the S/R 100:1 mixture once again there appeared to be an increase in the strain originally in the minority. The results obtained at the third and fourth months were surprising and were therefore repeated, but with similar results. The results with these mixtures are difficult to interpret. One explanation would be that the two strains grow at different rates. It is, however, possible that a transference of properties with regard to penicillin-sensitivity takes place.

The S/R 1:1 mixture was also subjected to X-irradiation of 1,000,000 r. to see whether this would affect one strain more than the other. The X-irradiated

mixture was then subcultured twice a week in Lemco broth for 6 months. It will be seen from Table 4 that the number of resistant colonies was diminished immediately, that it increased until the second month, after which it steadily decreased until at 6 months no resistant colonies were detected even when the culture was plated directly on to a penicillin ditch-plate. It seems probable that the sensitive strain was originally more susceptible to the lethal action of irradiation, but that serial subculture, as with the S/R 100:1 mixture not irradiated, led to the sensitive colonies, originally in the minority, eventually becoming predominant.

#### *Typing by bacteriophage*

Since the cultures used in these experiments were derived originally from single colonies, not single cells, the purity of eight strains was checked by comparing the phage reaction of parent cultures, the variants, and cultures obtained from penicillin-resistant descendant colonies. In all eight strains, the variants were of the same phage type as the parent, and as the penicillin-resistant daughter colonies.

#### *Colonial variations*

A number of variations in colonial appearance was observed during the course of these experiments, especially in cultures which had been in the laboratory for some time. The variant colonies were best seen when plates were incubated for 18–24 hr. and then left on the bench for a few days. Gross variations in colour and opacity occurred and were apparently favoured by repeated subculture, so that a single plate from a subcultured strain often consisted of typical golden, white, semi-transparent and transparent colonies. Size variations were also very common and were more marked in plates from the cultures that had been left on the bench without subculture, many consisting of a large number of small pin-point colonies with a few larger and more typical ones (Pl. 1, fig. 5). Another, less frequent variant occurring in several strains was a colony with an irregular and crenated edge, a rough or matt surface and a depressed centre (Pl. 1, fig. 6). All these colonial variants retained their capacity to clot citrated or oxalated plasma. Occasionally coagulase-negative colonies were encountered, but these were discarded as probable air contaminants. There was no association between colonial appearance and sensitivity to penicillin.

#### DISCUSSION

Studies on the permanence of resistance to penicillin of penicillinase-producing staphylococci have in many cases been invalidated by the assumption that the individual organisms making up a culture act uniformly in their behaviour to penicillin. Thus Blair, Carr & Buchman (1946) and Spink & Ferris (1947), as a result of testing the whole culture, claimed that cultures of penicillinase-producing staphylococci were permanently resistant to penicillin. But, since a few penicillinase-producing cocci may protect many penicillin-sensitive cocci in the culture from the action of penicillin (see Pl. 1, fig. 3), testing the whole culture does not give an accurate picture of what is really happening.

The present study makes it clear that penicillinase-producing strains of *Staph. pyogenes* tend to give rise to penicillin-sensitive variants when kept in the laboratory. It is, however, obvious that the method used is not accurate enough for much reliance to be placed on the actual number of variants obtained at any one time. Because single colonies, not single cells, were the starting-point for all experiments, it is possible that the original cultures consisted of a mixed population. The identity of phage-type of parent and variant colonies of eight strains, however, make it almost certain that at least in these strains, the penicillin-sensitive colonies were true variants of a penicillin-resistant parent.

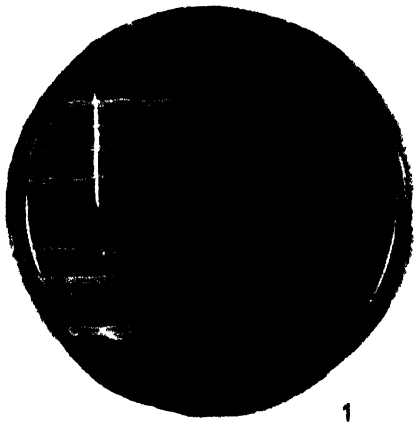
I have not yet found a method of accelerating the natural variation, but this line of investigation is being continued. Voureka (1948) claims to have rendered both types of penicillin-resistant staphylococci sensitive to penicillin by mixing them with other organisms, such as the Milne streptococcus. My results (Table 3) do not confirm this. Bennison & Schwabacher (1948) have also been unable to repeat Voureka's results. What happens when two strains of *Staph. pyogenes* are mixed is not clear. In both S/R 1:100 and S/R 100:1 mixtures there was an increase in the minority strain. It is, however, possible that the strain in the majority was giving rise to variant colonies.

Until we discover the mode of origin of penicillin-destroying strains of staphylococci, it is not possible to understand the mechanism whereby they give rise to penicillin-sensitive variant colonies. Three explanations have been put forward. The first is that this type of resistance is truly acquired by contact with penicillin. As pointed out by Luria (1947), this would represent an example of the inheritance of an acquired characteristic, and therefore is contrary to the 'general outlook of modern biological thought'. The second, suggested by Barber (1947*a*), is that a few strains of *Staph. pyogenes* have always been penicillin-resistant and are now increasing in frequency by a simple process of selection. This view is supported by the fact that so far it has not proved possible to produce this type of resistance to penicillin *in vitro*. The frequency, however, with which these strains yield penicillin-sensitive variants is against this hypothesis. The third view, first put forward by Demerec (1945) to explain the origin of 'acquired' penicillin-resistance *in vitro* by staphylococci, and later by Spink & Ferris (1947) as an explanation for the origin of penicillinase-producing strains, is that these resistant organisms arise originally as rare mutants independent of the presence or absence of penicillin. In the presence of the antibiotic, however, these mutants are favoured at the expense of the penicillin-sensitive parent and thus increased in frequency by differential selection. If this hypothesis be correct then the penicillin-sensitive variants recorded here must be regarded as reverse mutants. Their frequency, in view of the difficulty of demonstrating the direct mutation, is perhaps surprising, but as Luria (1947) says 'very mutable characters often present rates of reverse mutations higher than the direct mutation rates'.

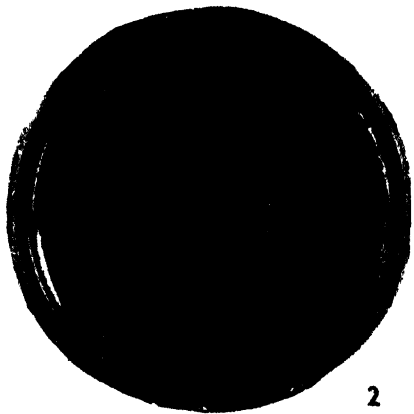
My thanks are due to Dr V. D. Allison for the phage-typing results, to Mr E. V. Willmott for the photographs, and to Dr A. Voureka for supplying me with a culture of the Milne streptococcus.



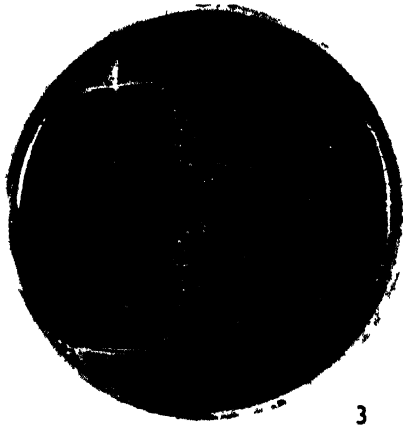




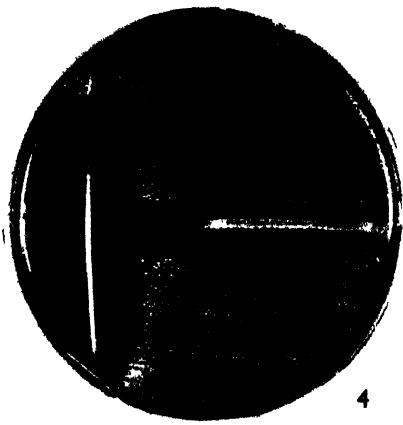
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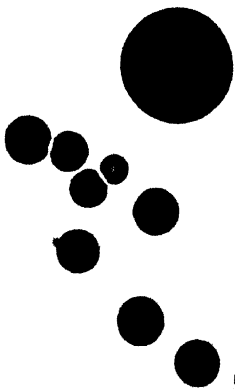
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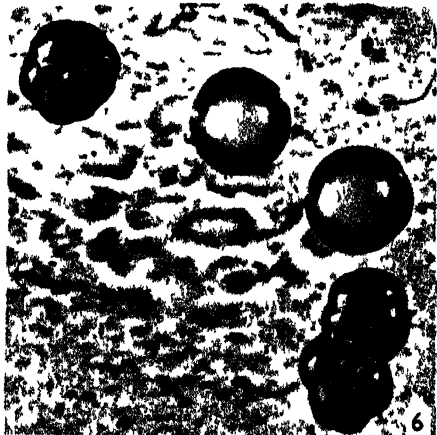
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4



5



6

Figs 1-6

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# EXPLANATION OF PLATE

## Penicillin ditch-plates ( $\times \frac{1}{2}$ )

- Fig. 1. Typical example of strain showing a mixture of penicillin-resistant and sensitive colonies. Central streak=Oxford staphylococcus.
- Fig. 2. Upper half plated with culture P6652, penicillin-resistant on primary isolation now sensitive, and lower half with the Oxford staphylococcus.
- Fig. 3. Strain giving nine out of fifty penicillin-sensitive colonies—mixture undetectable by this method. Central streak=Oxford staphylococcus.
- Fig. 4. Mixture of penicillin-sensitive and resistant staphylococci in proportion of 100 sensitive to 1 resistant. Note gradual fading out of sensitive colonies as penicillin is approached and large resistant colonies near penicillin. Central streak=Oxford staphylococcus.

## Colonial variations

- Fig. 5. One typical-sized colony and a number of small colony variants from same culture,  $\times 20$ .
- Fig. 6. Two typical smooth colonies of *Staph. pyogenes* and three 'rough' variants,  $\times 18$ .

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## The Fermentation of Trehalose by Yeasts and its Taxonomic Implications

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**SUMMARY:** In the classification of yeasts it is customary to use an infusion of bakers' yeast as the basal medium for fermentation tests. This extract frequently contains variable amounts of trehalose. A number of yeasts were observed to ferment yeast extract and trehalose. The fermentation of yeast extract is serious from a taxonomic point of view, since it gives the impression of positive fermentation of a sugar which actually may not be fermentable. Dilute yeast autolysate should be used as the basal fermentation medium since during autolysis trehalose is destroyed. The fermentation of yeast extract (without added sugar) is easily observed in Durham tubes by the collection of gas in the inserts, but when Einhorn fermentation tubes are used gas production is seldom apparent.

One culture (N-18) isolated from spoiled apricots, and identified as *Candida tropicalis* showed adaptive trehalose fermentation.

The ability of various yeasts to ferment trehalose was investigated, using 133 cultures, representing twenty genera and seventy-three species. Sixteen species representing seven genera fermented yeast extract and trehalose.

The fermentation of trehalose is worthy of consideration as a character for use in differentiating certain species of *Candida*, and perhaps other yeasts.

In identifying yeasts it is necessary to use a number of natural media the compositions of which are uncertain. Although the method of preparation may be well standardized, these natural media undoubtedly vary in composition from batch to batch. These variations were considered of sufficient importance by Bedford (1942) to warrant the use of a chemically defined medium rather than liquid wort for studying cell and film characteristics of *Hansenula* spp. The use of chemically defined media has not been extended to the other genera of yeasts, principally because most yeasts grow poorly in such media, unless fortified with growth factors. Stelling-Dekker (1931) used fermentation media prepared by adding 2% of a given sugar to a water extract of bakers' yeast. This method has also been used for a number of years in our laboratory.

During recent investigations it was observed that a strain N-18, isolated from home-canned apricots, and later identified as *Candida tropicalis*, appeared to ferment both maltose and lactose when the yeast-extract medium of Stelling-Dekker (1931) was employed in Durham fermentation tubes. In view of the observation of Kluyver (1931), that in general, yeasts fermenting maltose do not ferment lactose and vice versa, this finding seemed of interest. Custers's (1940) report, that *Brettanomyces claussenii* fermented maltose and lactose, is the only known exception to this rule. *B. claussenii* also ferments maltose and lactose when the auxanographic plate method of Lodder (1934) is used; but culture N-18 was found to ferment maltose and not lactose.

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In the past three years, occasional strains of yeast in this laboratory have fermented maltose strongly and apparently fermented lactose weakly. The majority of these strains were classed as belonging to the genus *Candida*. When these strains and strain N-18 were grown in yeast-extract medium without added carbohydrate, fermentation to a similar degree as that of lactose occurred, indicating the presence of a fermentable substance in the basal medium. To our knowledge only one similar observation has been reported in the literature. Sacchetti (1933) observed fermentation of plain yeast extract by certain *Torulopsis* species, but he did not attempt to explain the phenomenon. It appears quite possible that in the past fermentation tests with other common sugars have incorrectly been termed positive in cases when gas might have been produced from the plain yeast extract instead of the added sugar.

Bakers' yeast is known to contain variable amounts of the disaccharide trehalose, sometimes as high as 14 % of the dry-weight (Myrbäck & Örtengren, 1936). Trehalose was first found in American bakers' yeast by Koch & Koch (1925), and later in Dutch bakers' yeast by Boesecken (see Kluyver & van Roosmalen, 1932), in French bakers' yeast by Tanret (1931), again in America by Steiner & Cori (1935) and in Sweden by Myrbäck (1937). Brandt (1941) has shown that although bakers' yeast grown under aerobic conditions stores considerable quantities of trehalose, it stores none under anaerobic or semi-anaerobic conditions. It seemed likely that the trehalose of bakers' yeast might be extracted during the preparation of yeast extract and would be responsible for the observed fermentation of plain yeast extract. This paper deals with the nature of the fermentable material in bakers' yeast extract (Stelling-Dekker), the types of yeast which are able to ferment it and its possible significance in yeast taxonomy.

## EXPERIMENTAL

*Methods.* Two basal media were employed for fermentation studies, yeast extract and yeast autolysate. The yeast extract (Stelling-Dekker, 1931) was prepared by autoclaving a suspension of 200 g. of bakers' yeast in 1 l. water for 15 min. under 15 pounds pressure, and filtering, first while hot and again when cold. The extract may be easily clarified with fresh egg-albumin, or with a filter-aid. The yeast autolysate was made by incubating equal weights of bakers' yeast and water at 55° for 72 hr., then bringing the mixture to a boil and filtering with the aid of 'filter-cel'. The yeast extract was used undiluted, and the autolysate diluted with nine parts of water. Except where noted, fermentations were carried out in small Durham fermentation tubes. When required, 2 % of the desired carbohydrate was added to the basal medium.

### *Isolation and identification of the fermentable substance in the yeast extract*

In order to determine the nature of the fermentable substance present in yeast extract prepared from bakers' yeast, a vacuum concentrate (5:1) of the extract was fractionated with increasing concentrations of ethanol and the precipitates formed were each made up to original volume with water. The last

fraction, containing the material soluble in 70 % (v/v) ethanol, was evaporated *in vacuo* and the residue made up to volume, as were the precipitates. A sample of each of the fractions was used as a medium for fermentation tests with strain N-18. Only the fraction soluble in 70 % ethanol produced gas, indicating that the fermentable substance was probably a simple carbohydrate.

The unknown material was obtained in quantity from vacuum-dried yeast extract by the methods for the isolation and purification of sugars as outlined by Morrow & Sandstrom (1935). The material was non-reducing, and was proved by specific rotation and other characteristics to be identical with trehalose. Strain N-18, and other strains forming gas in yeast extract, fermented the isolated trehalose, thus indicating that it was responsible for the gas production in yeast extract.

*Effect of the type of fermentation apparatus.* That previous workers using yeast extract as the basal fermentation medium (Stelling-Dekker, 1931; Lodder, 1934; Diddens & Lodder, 1942) have not reported the presence of a fermentable substance or the apparent combined fermentation of maltose and lactose may be due to the type of fermentation tube employed. The Dutch workers use Einhorn tubes for routine work. To clarify this point of difference, comparative fermentation tests with a number of trehalose-fermenting strains were made on the same batch of yeast-extract medium in Einhorn tubes, large Durham tubes (Henrici, 1941) and small Durham tubes.

There was gas production in all Durham tubes but none in the Einhorn tubes. Mixing of the contents of the Einhorn tubes after several days' growth as recommended by the Dutch workers, in order to give greater sensitivity, resulted in only two positives out of sixteen strains which were positive in Durham tubes. These findings confirm the above hypothesis, indicate the lesser sensitivity of the Einhorn tubes, and suggest that when Durham tubes are used another basal fermentation medium should be employed.

Neither time nor method of heating yeast in water during the preparation of yeast extract appear to be important factors, since steaming at 100° for 10 min. or autoclaving at 120° for 5, 10, 15, 25, or 40 min., all gave similar results when fermentation tests were made with the resultant extracts. The concentration of the extract is important, since as a result of differences in manufacture and yeast strain used, the trehalose content of bakers' yeast varies greatly. In one instance, yeast extract (Stelling-Dekker) showed gas production in 72 hr. with strain N-18. When this yeast extract was diluted to half strength, 4 days were required for gas production, whereas growth but no gas production was noted when the dilution was greater than 1/4. Concentration of the original extract, on the other hand, shortened the time taken for gas formation to appear.

#### *Adaptive fermentation of trehalose*

When strain N-18 was grown on yeast-extract agar instead of wort agar before inoculation into yeast-extract fermentation tubes, gas production was greater and more rapid, suggesting an adaptive mechanism. Similar results were obtained when the organism was grown on a medium containing trehalose.

Experiments conducted with the Warburg respirometer in anaerobic con-

ditions and the washed-cell technique confirmed our findings (see Fig. 1). Strain N-18 was grown on a medium containing 10% of yeast autolysate and 1% added glucose or trehalose in Kollé flasks at 30° for 24 hr. Strain N-18 grown in 1% glucose was unable to adapt itself to trehalose fermentation under

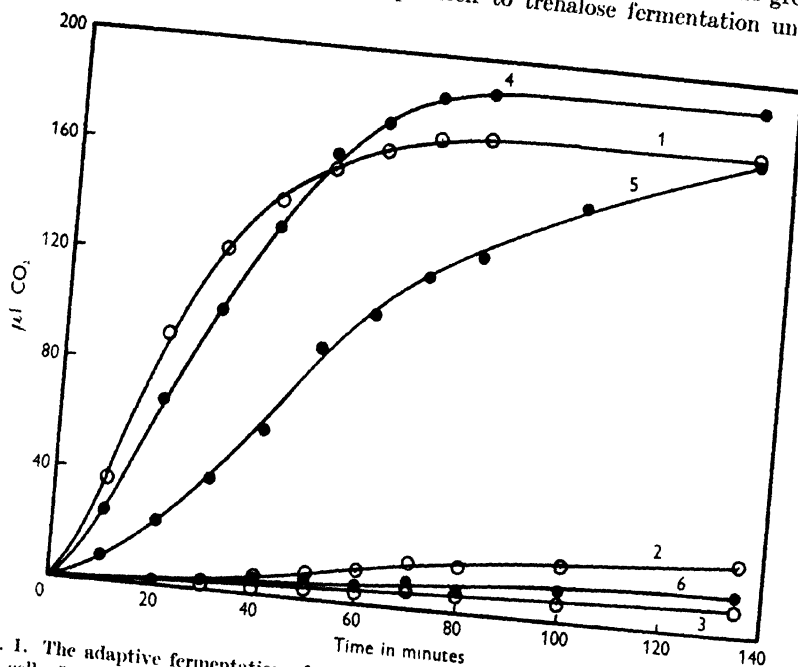


Fig. 1. The adaptive fermentation of trehalose by *Candida tropicalis*. (1) Glucose-grown cells, 5  $\mu$ mol. of glucose; (2) glucose-grown cells, 2.5  $\mu$ mol. trehalose; (3) glucose-grown cells, endogenous; (4) trehalose-grown cells, 5  $\mu$ mol. glucose; (5) trehalose-grown cells, 2.5  $\mu$ mol. trehalose; (6) trehalose-grown cells, endogenous. Temperature 30°; v. 30-KH<sub>2</sub>PO<sub>4</sub> buffer.  $\circ$ — $\circ$ , glucose-grown cells;  $\bullet$ — $\bullet$ , trehalose-grown cells.

anaerobic conditions within 2½ hr. after contact with the new substrate. On the other hand, when the culture was grown in the presence of trehalose, it fermented the trehalose at almost the same rate as it did glucose. Essentially, similar results were obtained using the van Iterson-Kluyver fermentometer (see Stelling-Dekker, 1931), in which conditions are also anaerobic and little or no cell multiplication occurs. With this technique the observations were extended over a period of about 24 hr.

This yeast N-18 is, as far as we know, the first reported to show a distinct adaptive trehalose fermentation. Previously it was indicated (Rhoades, 1941) that a beer yeast (of doubtful identity, since it apparently could not ferment maltose under any conditions) and a strain of *Saccharomyces cerevisiae* fermented trehalose when the cells were grown on galactose, maltose, melzitose or  $\alpha$ -methylglucoside, but not when grown on glucose. Rhoades used Lindner's well-slide technique to test fermentation. His paper, however, leaves the impression that it would be desirable to repeat some of the experiments before accepting the conclusions.

*Recommended basal medium*

The problem of preparing a basal medium free of fermentable sugar was overcome by using a diluted yeast autolysate (as described above) rather than a water extract. Myrbäck & Örtenblad (1937) demonstrated the enzyme trehalase in yeast autolysate and on the cell debris, and showed that the glucose formed by hydrolysis was fermented during autolysis. This explains why the stored trehalose disappears during autolysis of baker's yeast.

Another method of obtaining a basal yeast-extract medium free of trehalose is to use brewers' yeast instead of bakers' yeast. Müntz (1876) sought trehalose in brewers' yeast because of its presence in other fungi, but was unable to detect it. Myrbäck & Örtenblad (1937) also found no trehalose in brewers' yeast, an observation we were able to confirm with two different commercial samples of American brewers' yeast. The extracts of these samples failed to show gas production with strain N-18, unless a fermentable sugar was added.

*Fermentation of trehalose and yeast extract by various yeasts*

The study of the trehalose fermentation was extended in the hope that some of the results might have taxonomic application. The first trials indicated that only a few yeasts belonging to the genus *Candida* were strong trehalose fermenters, although none of the stock cultures tried compared in rate with strain N-18 (identified as *C. tropicalis*).

If the use of trehalose fermentation would help the classification of *Candida* species or other yeasts it would well justify the addition of another sugar to those already used in routine fermentation and utilization tests. Several workers have tested yeasts and other fungi for trehalose fermentation. Bau (1899) reported that trehalose is fermented by *Saccharomyces ellipsoideus*, *S. pastorianus* and *Monilia candida*, but very little by the 'Apiculate yeasts' or *Schizosaccharomyces*. Lindner (1887) found other yeasts capable of fermenting trehalose, namely *Monilia candida*, *Saccharomyces anomalus*, *Sacch. pastorianus*, *Sacch. ellipsoideus* and several unnamed bottom yeasts. He reported that only five out of thirty-seven cultures of wild yeast were unable to ferment trehalose. Unfortunately, it is not even possible to speculate as to the identity of these yeasts. Kluyver & Roosmalen (1932) showed that *Torula dattila* Kluyver, and *Saccharomyces cerevisiae* Rasse van Delft fermented trehalose slowly, but *Torula monosa* Kluyver and *Torula lactosa* Kluyver were unable to do so. Myrbäck & Örtenblad (1936) obtained variable results in tests on the fermentation of added trehalose by bakers' yeast; in some cases a slow fermentation occurred, whereas in others no gas was observed. On the other hand, dried yeast preparations made from samples of the same bakers' yeast fermented trehalose very well, indicating the presence of the enzyme system necessary for the fermentation of this sugar. Brewers' yeast (bottom yeast) was found to ferment added trehalose regularly but slowly.

We have checked the fermentation of yeast extract (autoclaved equal weights of water and bakers' yeast) by 133 cultures representing twenty genera and

seventy-three species. The fermentation of added trehalose (1%) in diluted yeast autolysate, prepared as previously described, was also examined. Most tests were repeated at least once. Of the large number of cultures used, only sixteen species, representing seven genera of yeasts, were positive for the fermentation of yeast extract and trehalose. In all cases, when the fermentation of plain yeast extract was positive, gas production in the autolysate plus added trehalose was also positive. There were some instances, however, when gas production occurred in the trehalose tubes but not in the yeast-extract tubes. When these tests were repeated with a more concentrated yeast extract and longer incubation period, gas production occurred in the yeast-extract tubes as well as the trehalose tubes. These fermentation tests are discussed in more detail in connexion with the various genera investigated.

*Genus Candida.* If trehalose fermentation has any value as a character in yeast classification it should be so with certain species in the genus *Candida*. This is particularly true of *C. albicans* and *C. tropicalis*, the definitions of which (Diddens & Lodder, 1942) are quite broad and inclusive. Thirteen cultures of *C. albicans*, from various sources, were tested and seven produced gas in yeast extract as well as in the tubes with autolysate + trehalose. Six cultures were non-fermentative, two being cultures of Mackinnon's (1946) normal and variant strains. The variant, which was considered negative, showed a questionable fermentation in 13 days.

Two of three cultures of *C. tropicalis* gave positive fermentations. The identity of the culture which gave a negative fermentation is in our opinion questionable. The fermentation of trehalose by *C. tropicalis* is of particular interest, since strain N-18 and several other organisms isolated by us from cheese, olives, grapes, dates and soil, and capable of fermenting trehalose, are cultures of *C. tropicalis*, or are very similar to it. These are organisms that appeared to ferment both maltose and lactose in yeast-extract media, whereas actually they can ferment only maltose.

Two strains of *C. stellatoidea* and one stated to be *C. deformans* (probably *C. lipolytica* or *C. zeylanoides*), were positive for yeast extract, and trehalose. The following organisms grew, but failed to produce gas in either yeast extract, or autolysate plus trehalose: fourteen strains of *C. krusei*; five of *C. chalmersi* (*C. parapsilosis*); two of *C. pseudotropicalis*; and one strain each of *C. suaveolens* (*C. humicola*), *C. guilliermondii*, *C. flareri* (*C. intermedia*), *C. intermedia*, *C. brumpti*, *C. zeylanoides* var. *macroglossia*, *C. parakrusei* (*C. parapsilosis*) and *C. pelliculosa*.

*Genus Saccharomyces.* Sixty-three strains representing eleven species of this genus were tried. Only five strains were positive. In this genus the strongest fermenter of both yeast extract and trehalose was a hybrid, *S. carlsbergensis* × *S. cerevisiae* obtained from Dr C. Lindgren. This is of interest since the four strains of *S. carlsbergensis* tested were negative and of the thirty-two strains of *S. cerevisiae* used, only one received from São Paulo, Brazil, was weakly positive. One strain each of *S. italicus* and *S. cerevisiae* Rasse *fulliensis* showed positive fermentation. In 1946 a culture of *Saccharomyces*, showing ability to ferment both maltose and lactose, was isolated from spoiled fruit.



Unfortunately the culture was lost, but in all probability the unusual fermentation characteristic of this yeast was likewise an artefact.

The following were negative for the fermentation of yeast extract and trehalose: eight strains of *S. cerevisiae* var. *ellipsoideus*; four of *S. tubiformis*; three of *S. oviformis*; two of *S. fragilis*; two of *S. chodati*; and one strain each of *S. exiguus*, *S. pastorianus*, *S. pyriformis* and *S. thermantitonum* (*S. cerevisiae* var. *ellipsoideus*).

**Subgenus Zygosaccharomyces.** Seventeen strains representing twelve species were used. Only two strains, both *Z. pini*, were weakly positive. These strains were negative after 5 days in yeast extract prepared according to Stelling-Dekker (1931); when a much more concentrated extract and twelve days' incubation were used they were positive. Organisms not showing fermentation were: two strains of *Z. barkeri*; two of *Z. japonicus* var. *Soya*; two of *Z. bisporus*; and one strain each of *Z. marxianus*, *Z. nadsonii*, *Z. pastori*, *Z. acidifaciens*, *Z. globiformis*, *Z. richteri* and *Z. lactis*.

**Genus Hansenula.** Of thirty-seven strains representing seven species only five strains were fermenters of trehalose. After 10 days of incubation only one of six strains of *H. anomala* and three out of nine strains of *H. anomala* var. *longa* were positive. The results obtained with the variety 'longa' were quite variable, depending on the concentration of yeast extract and period of incubation. Similar results were obtained with a single strain of *H. cifferi*. Those showing no fermentation were seven strains of *H. anomala* var. *spherica*; six of *H. saturnus*; three of *H. subpelliculosa*; and one strain each of *H. schneggii*, *H. lambica* and *H. anomala* var. *heteromorpha*.

**Genus Debaryomyces.** Only three strains were tried but two (*D. globosus* and *D. matruchoti*) were quite strongly positive, whereas *D. tyrocola* was negative.

**Torulaspora.** A single strain, *T. fermentati* was positive, and one of *T. delbrücki* negative.

**Nematospora.** *N. coryli* (one strain) was one of the strongest gas-producers in yeast extract as well as in trehalose.

**Torulopsis.** Eight strains representing eight species were tried. Only *T. alactosa* (*T. holmii* Rasse Delft) was unquestionably positive. *T. dattila* produced a few small bubbles. Kluyver & van Roosmalen (1932) also reported this to be a slow fermenter of trehalose. Those showing no fermentation were: two strains of *T. pulcherrima* (*C. pulcherrima*); *T. fermentans*; *T. kefir*; *T. monosa*; *T. californicus*.

**Genus Brettanomyces.** A single strain identified as *B. bruxellensis* failed to show any fermentation. However, two strains isolated from Irish beer and tentatively identified as *Brettanomyces* species fermented both yeast extract and trehalose rapidly.

**Other genera.** All other strains tried were negative. These were: *Schizosaccharomyces octosporus* and *Sch. pombe*; *Saccharomycoides ludwigii*; *Endomycopsis selenospora*, *E. albicans*, *E. capsularis* and *E. lindneri*; *Endomyces magnusii*; *Schwanniomyces occidentalis*; *Pichia chodati*, *P. fermentans*, *P. kluyveri* and *P. membranaefaciens*; *Zygopichia chevalieri*; *Zyghansenula californica*;

*Nadsonia fulvescens*; *Eremothecium ashbyi*; *Schizoblastosporion starkeyi-henrici* and *Kloeckera africana* and *K. lindneri*.

The authors are indebted to Dr Carl Lindegren for the culture of *Saccharomyces cerevisiae* × *carlsbergensis*, to Dr Juan Mackinnon for the normal and variant strains of *Candida albicans*, and to Dr L. Wickerham for several of the other strains used.

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## The Susceptibility of Viruses to Ethyl Ether

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**SUMMARY:** We have examined by a uniform technique the sensitivity of twenty-five viruses to ethyl ether. Evidence of the sensitivity of ten other viruses is available from the literature. Viruses seem to be either very sensitive or highly resistant. Of the viruses pathogenic to animals, most of the resistant ones are either in the pox group or amongst the very small viruses. The results may prove of use to workers who at times need to separate one virus from mixture with others or with bacteria. The findings may also be of value in any attempt at virus classification.

Many records attest that some viruses, notably those of vaccinia and poliomyelitis, are highly resistant to inactivation by ethyl ether. A recent paper by Sulkin & Zarafonitis (1947) reports that three insect-borne encephalitis viruses (St Louis, Western and Eastern equine encephalomyelitis viruses) are in contrast highly susceptible; that of rabies was found, like poliomyelitis, to be resistant. We were led to test the action of ether on a variety of viruses, to determine how widespread in the group ether-resistance might be.

### *Methods*

The application of a single simple technique to these viruses was important. We employed pure anaesthetic ethyl ether (May & Baker, London); this was free from demonstrable peroxides and aldehydes. Tissue suspensions or gradocol membrane filtrates of them were made up in Hartley's broth containing 10 % horse-serum; 20 % ether by volume was added and the mixture placed in a screw-capped bottle. Adhesive tape was bound round the cap to reduce any risk of loss of ether. After being shaken, the bottle was held at  $+4^{\circ}$  for 18–24 hr. Control specimens were treated similarly but without ether. In many instances, some opalescence or a little readily dispersed precipitate appeared in ether-treated specimens. As a rule little or no layering of ether above the virus suspension was visible after overnight contact. Ether was removed by pouring the specimen into an uncovered Petri dish and allowing evaporation to occur at room temperature; this was rapid, no smell of ether being detected after 10 min. Control and treated specimens were then titrated in the appropriate way in susceptible animals or fertile eggs. Any variation from this technique is recorded in the text.

### RESULTS

The viruses tested were found, in general, to be either wholly resistant to this ether treatment or else highly susceptible. The susceptible viruses mostly had their titre reduced a thousand-fold or more. One or two, however, infected initially only in low dilutions, and an estimate of the amount of inactivation was not possible. The viruses tested will be discussed *seriatim*. In almost all cases several tests were carried out and the results were concordant. In the

following account, the viruses we have tested ourselves are numbered; others, the ether sensitivity of which is mentioned in the literature, are referred to but not numbered. The factors by which the titres of the various virus preparations were diminished are recorded in Table 1. In the ether-resistant group there was no demonstrable drop in titre.

#### Group I. *Psittacosis-lymphogranuloma* group

(i) *Mouse pneumonitis* (Nigg, 1942*a*). This virus, obtained from our own laboratory mice, was titrated by inoculating centrifuged lung-suspensions intranasally into mice, which were killed 7 days later. Material producing extensive lesions but no deaths at  $10^{-5}$  dilution, was, after ether treatment, inactive even when undiluted; 25 % ether was used.

(ii) *Feline pneumonitis* (Baker, 1944). A virus, provisionally identified thus, was obtained from a cat suffering from chronic catarrh; it was pathogenic for mice and was titrated as for the last virus. Material producing pneumonia at  $10^{-4}$  dilution was inactive even undiluted after ether treatment.

*Psittacosis* (Gordon, 1930-1) and *Lymphogranuloma venereum* viruses (Nigg, 1942*b*) are reported to be susceptible to ether.

#### Group II. *Pox* group

(iii) *Vaccinia virus* of Salaman's rabbit dermal strain was used in the form of centrifuged suspensions of infected testis and of washed elementary bodies from rabbit skin. Dr A. S. McFarlane kindly provided the latter. The virus was titrated by intradermal inoculation of shaved rabbit skin. Material active at  $10^{-6}$  had an identical titre after treatment. Tests on several rabbits gave the same results.

(iv) *Infectious ectromelia*. Filtrates of infected mouse lung of the Moscow strain of virus were titrated by intraplantar inoculation of mice. These were observed for the development of local lesions and death. Material used infected mice in a  $10^{-5}$  dilution, both before and after ether treatment. In one experiment, a little (< 10-fold) drop in titre was seen if lesions were used to determine the end-point, but if death was the criterion, the etherized virus was the more active.

(v) *Contagious pustular dermatitis*. Washed elementary bodies sent us by Mr F. Blakemore were titrated by rubbing falling dilutions into scarified areas of rabbits' skins. Definite lesions developed but their occurrence was rather irregular, so that one had no confidence in reading an end-point. It appeared, however, that the ether treatment affected the titre little or not at all.

*Goat-pox* and *sheep-pox* are reported by Bennett, Horgan & Haseeb (1944) to be resistant to ether.

#### Group III. *Other relatively large viruses (probably heterogeneous)*

(vi) *Rabbit myxoma*, given us by Dr E. W. Hurst, was used as a centrifuged suspension of infected testis and titrated by intradermal inoculation into rabbits. The titre, in three rabbits, fell respectively from  $10^{-4}$  to  $10^{-1}$ ,  $10^{-5}$  to  $10^{-2}$ ,  $10^{-5}$  to  $10^0$ .

(vii) *Rabbit fibroma* (OA strain), similarly prepared and titrated, fell in titre respectively from  $10^{-2}$  to a negative at  $10^0$  and from  $10^{-4}$  to  $10^{-1}$ . The occurrence of generalized lesions obscured the readings after about the fifth day.

(viii) *Grey-lung virus* (Andrewes & Glover, 1945). This virus of mice was titrated in the same way as was Nigg mouse-pneumonitis. Suspensions gave extensive lung lesions at  $10^{-4}$  and even this was not an end-point; undiluted ether-treated virus was inactive.

(ix) *Herpes simplex*. A fairly recently isolated (Syatt) strain was passed through rabbit testes. It gave lesions on intradermal inoculation of rabbits only to a titre of 1:100 or 1:10. This activity was quite abolished by ether treatment. On passage through mouse-brains it attained an intracerebral titre in mice of  $10^{-2}$ ; again etherized virus was inactive.

(x) *B. virus* (Sabin, 1934). This virus, which is related to that of herpes, was sent by Dr E. W. Hurst. A centrifuged suspension of infected rabbit brain was titrated intradermally in rabbits. This produced lesions only when undiluted or diluted 1/10. Etherized virus was inactive.

Rabies virus was found by Sulkin & Zarafonitis (1947) to be ether-resistant; they quote work, however, which suggests that different strains differ in ether resistance.

#### Group IV. *Influenza group*

(xi) *Influenza A* (PR8 strain). Infective allantoic fluid was titrated by allantoic inoculation into groups, usually of three eggs; fluids from these were tested for haemagglutinating activity. In three experiments titres dropped as a result of ether treatment, from  $10^{-9}$  to  $10^{-4}$ ,  $10^{-8}$  to  $10^{-2}$ ,  $10^{-10}$  to  $10^{-2}$  respectively. Mouse-lung filtrate titrated similarly in eggs dropped from  $10^{-5}$  to  $10^0$ . Material, whether derived from eggs or mice, gave similar results when titrated by intranasal inoculation of mice. The drops in titre obtained were from  $10^{-4}$  to  $<10^0$  (mouse-lung filtrate); and  $>10^{-6}$  to  $10^{-1}$  and  $10^{-5}$  to  $10^{-1}$  (allantoic fluid).

(xii) *Influenza B* (Lee strain). Results with Influenza B were much the same as with A. Ether treatment of allantoic fluid reduced the titre about 100-fold when titrations were made in mice. With allantoic titrations ether treatment caused a drop from  $10^{-7}$  to  $10^{-1}$ .

(xiii) *Mumps*. Infected allantoic fluid was titrated allantoically, groups of three eggs being used for each dilution. The drop in titre produced by ether treatment was from  $10^{-7}$  to  $10^{-2}$ .

(xiv) *Fowl plague*. Virus in infected allantoic fluid was titrated in eggs as described for influenza. The drop in titre in a representative experiment was from  $10^{-7}$  to  $10^{-4}$ —a 1000-fold.

(xv) *Newcastle-disease virus* was similarly examined and the titre fell  $10^5$ -fold, from  $10^{-8}$  to  $10^{-3}$ .

#### Group V. *Tumour viruses*

The Rouse sarcoma and papilloma viruses are considered together here, though it is unlikely that they are related.

(xvi) *Rous sarcoma*. Filtrates of tumours were titrated by intramuscular

injection into the breasts and legs of chicks about 6 weeks old; the tumours produced by control filtrates of any one strength were very uniform in size in the six chicks in the experiment. Control material was active at a dilution of 1:1000. After ether treatment tumours of comparable size were produced only by undiluted filtrate.

(xvii) *Rabbit papilloma*. The warts tested had been kept in glycerol for 12 years and extracts only produced warts on scarification of rabbits' skins when applied undiluted. The ether-treated virus extracts proved, however, equally active with controls. (Drop in titre: nil).

#### Group VI. *Arthropod-transmitted viruses*

(xviii) *Yellow fever*. A neurotropic virus was received from Dr D. J. Bauer; centrifuged suspensions of infected brains were inoculated intracerebrally in mice and produced deaths up to a dilution of  $10^{-3}$ . Virus treated with 25 % ether only did so when given undiluted. (Drop in titre  $10^3$ .)

Owing to lack of accommodation in our isolation rooms we carried out only single experiments with the following two viruses.

(xix) *Rift-valley fever*. Liver suspensions from an infected mouse, again sent by Dr D. J. Bauer, were titrated intraperitoneally in mice. Control material infected to a titre of  $10^{-4}$ , ether treated (20 %) undiluted, doubtfully at 1:100.

(xx) *Louping-ill*. Infective mouse brain, titrated intracerebrally in mice, was active at  $10^{-7}$ . Etherized virus infected only to a dilution of  $10^{-5}$ . This virus was provided by Dr D. G. ff. Edward. The drop in titre of not more than  $10^2$  is less than that of other viruses in the ether-sensitive groups. It is the smallest virus in this group and may show a transition in properties to group VII.

(xxi) *Lymphocytic chorio-meningitis*. This virus is potentially arthropod-transmitted, though this is probably not the normal route. Its similarity in size to the others makes it, however, conveniently considered in this group. Since many infected mice do not die, a sharp end-point in titrations of this virus is notoriously difficult to achieve. The WE strain we used was provided by Dr F. O. MacCallum. Brain suspensions were titrated intracerebrally in mice and were active at  $10^{-6}$  dilution. Ether-treated suspensions were inactive.

*The viruses of St Louis, eastern and western equine encephalomyelitis* were reported by Sulkin & Zarafonetis (1947) to be inactivated by contact with 10 or 20 % ether for 2 hr. at  $37^\circ$ . The drop in titre was of the order of  $10^5$ -fold.

#### Group VII. *Very small viruses*

(xxii) *Mouse encephalomyelitis* (FA strain, Theiler & Gard 1940). This virus differs in some properties from Theiler's original (TO) mouse encephalomyelitis. Mouse-brain suspensions were, in our hands, infective for mice intracerebrally to a titre of  $10^{-5}$  and equally so after ether treatment. We thus confirmed previous reports by Theiler & Gard (1940) and others. (Drop in titre: nil.)

There are plentiful reports since that of Taylor & Amoss (1917) of the resistance of human poliomyelitis virus to ether. Sulkin & Zarafonetis (1947) found that

the Lansing strain of poliomyelitis virus mixed with ether (to give a final concentration of 95 % ether and  $10^{-3}$  virus) and shaken for 2 hr. at  $37^{\circ}$ , showed no loss of infectivity.

*Foot-and-mouth disease*: Dr I. A. Galloway kindly allows us to quote some experiments carried out at the Virus Research Institute, Pirbright. Ether, 33.3 %, was added to potent virus in serum (titre  $10^{-4}$  and  $10^{-3}$  in two experiments). After short contact at  $-22^{\circ}$  the ether was removed and the virus found to be still potent. The ether-treated virus was not titrated.

#### Group VIII. *Bacterial viruses*

(xxiii-xxv) Three phages of different sizes, the relatively large staph K, the intermediate coli-phage C36 and the very small dysentery phage S13 were treated with ether under the same conditions as with animal viruses. The titres of the three were  $4 \times 10^8$ /ml.,  $8 \times 10^7$ /ml. and  $2 \times 10^7$ /ml. before ether treatment, and no appreciable drop in titre was produced by ether in any instance.

*Tobacco-mosaic virus*. A report by Allard (1916) suggests that tobacco-mosaic virus is probably ether resistant.

#### *Conditions affecting the action of ether*

*Medium*. Several viruses, notably influenza and herpes, were tested as suspensions of tissues derived from different hosts and no evidence was obtained suggesting that such differences affected the results. In some experiments with influenza, it appeared that the titre of material diluted a 100-fold in saline was diminished more by etherization than that of a similar suspension in serum broth, but the differences were not large. Ectromelia and FA viruses and phage C36 diluted in saline were still wholly resistant to ether. We carried out no experiment on the effect of ether on purified viruses.

*Temperature*. McFarlane (1942) has shown that lipid can be extracted from plasma by ether treatment below  $-20^{\circ}$ , where extraction at higher temperatures is less complete. We found that the drop in titre caused by ether treatment of influenza A, mumps and fowl plague was greater at  $-76^{\circ}$  than at  $+4^{\circ}$ ,  $10^6$ -fold as against  $10^4$ -fold in an experiment with influenza A titrated in eggs,  $10^6$ -fold as against  $10^5$ -fold with mumps, and  $10^6$ -fold against  $10^3$ -fold in a trial with fowl plague. The results were not regular and in one trial with Newcastle disease virus no difference was detected. The resistant viruses vaccinia, ectromelia, FA-encephalomyelitis, rabbit papilloma and phage C36 were still fully resistant when treated at  $-76^{\circ}$ .

*Ether concentration*. The tissue extracts studied were approximately saturated by the 20 % of ether added. The few experiments we carried out with weaker (10 %) or stronger (25-40 %) ether indicated that many sensitive viruses were definitely affected even by 10 % ether, and that increasing the amount of ether did not succeed in inactivating the resistant ones. Such results accord with those of Sulkin & Zarafonitis. One would not expect addition of ether above the saturation point to affect the results.

## DISCUSSION

In Table 1 viruses are arranged vertically, roughly according to size, and to right or left according to their resistance to ether. The ranges of size were allotted in consultation with our colleague Dr W. J. Elford, and are largely based on measurements reviewed by him (Elford, 1988). Study of such an arrangement

Table 1. *Viruses arranged according to ether sensitivity and, roughly, size*

| Group and size range   | Ether-sensitive  | Group and size range                                       | Ether-resistant  |
|------------------------|--|--|--|
| I. 175–330 m $\mu$ .   | Feline pneumonitis [ $> 10^4$ ]<br>Mouse pneumonitis [ $> 10^5$ ]<br>(Psittacosis)<br>(Lymphogranuloma venereum)   | II. 150–200 m $\mu$ .                                      | Vaccinia<br>Infectious ectromelia<br>? Contagious pustular dermatitis<br>(Goat-pox)<br>(Sheep-pox)<br>(Rabies) |
| III. 150–200 m $\mu$ . | Grey lung virus [ $> 10^4$ ]<br>Rabbit myxoma [ $10^3$ – $10^5$ ]<br>Rabbit fibroma [ $10^3$ ]<br>Herpes simplex [ $> 10^3$ ]<br>B. Virus [ $> 10$ ]   |  |  |
| IV. 60–120 m $\mu$ .   | Influenza A [ $10^4$ – $10^8$ ]<br>Influenza B [ $10^2$ – $10^6$ ]<br>Mumps [ $10^3$ ]<br>Fowl plague [ $10^3$ ]<br>Newcastle disease [ $10^5$ ]   |  |  |
| V. 75 m $\mu$ .        | Rous sarcoma [ $10^3$ ]  | 30–50 m $\mu$ .  | Rabbit papilloma   |
| VI. 20–35 m $\mu$ .    | Lymphocytic choriomeningitis [ $> 10^6$ ]<br>Rift valley fever [ $10^3$ ]<br>(St Louis encephalitis [ $10^5$ ])<br>(Western equine encephalomyelitis [ $10^5$ ])<br>(Eastern equine encephalomyelitis [ $10^5$ ])<br>Yellow fever [ $10^3$ ]<br>Louping-ill [ $10^3$ ] | VIII. 50–75 m $\mu$ .<br>20–30 m $\mu$ .<br>8–12 m $\mu$ . | Bacteriophage Staph. K<br>Bacteriophage C36<br>Bacteriophage S13   |
|                        |  | VII. 8–15 m $\mu$ .  | (Foot-and-mouth disease<br>(Human poliomyelitis)<br>Mouse encephalomyelitis<br>(FA)                            |

Viruses in round brackets have been examined by other workers, usually by techniques rather different from our own. The factor by which the titre of each ether-sensitive virus preparation was diminished is cited in square brackets.

may be helpful in enabling one to approach a little nearer to a classification of viruses. Many viruses which we had no opportunity to study will not fall conveniently into any of the rough groupings we have used in this paper and in the table. There is an interesting and perhaps significant correlation between our results and those of Wilson Smith (1939) on the bile-salt sensitivity of viruses. He found by experiment or from records in the literature that the following (all ether-sensitive) were inactivated by bile salts: influenza A, louping-ill, Rous sarcoma; whereas poliomyelitis, foot-and-mouth disease, vaccinia and ectromelia, all ether-resistant, also resisted bile salts. Rabies however, is said to be ether-resistant, yet sensitive to bile salts. It was



certainly resistant in Sulkin & Zarafonitis's tests, but according to Remlinger (1919) is slowly inactivated when infected brain is immersed in pure ether. Psittacosis, on the other hand, according to Burnet & Lush (1940), is resistant to bile-salts, though we find it ether-sensitive. On the whole the viruses which we have found to be ether-resistant are 'tough' viruses, viruses which can be left at room temperature without fear that they will quickly lose activity. The ether-sensitive ones are, on the whole, more delicate.

A few discrepancies in our results must be mentioned. Influenza A and B virus were invariably reduced  $10^4$ -fold or more in titre by ether when titrated allantoically: when, however, mouse inoculation was used, a few of our earlier experiments, especially with Influenza B virus of low titre, showed little or no inactivation. Again, vaccinia was apparently wholly resistant whenever titrated intradermally in rabbits, but, according to our colleague Dr K. R. Dumbell, showed a drop in titre after ether treatment when titrated by counting pocks on the chorioallantoic membrane. Using this same technique Dr F. O. MacCallum found a similar effect of ether on variola. As yet, our efforts to account for these discrepancies have been unsuccessful. Other workers seem agreed that influenza viruses are ordinarily ether-sensitive, while vaccinia is not.

One cannot readily correlate our findings with what is known of the chemical composition of viruses. Viruses known to contain both protein and lipids occur in both sensitive and resistant categories. Possibly the arrangement of the virus-components is important—whether or not, for instance, lipid occurs on the surface of the particles. One cannot easily exclude the possibility that in apparent inactivation by ether, aggregation at times plays a part. Such aggregation has, however, been equally apparent in preparations of sensitive and resistant viruses. Aggregates formed have been largely broken up by gentle shaking. Moreover, the complete inactivation or 1000-fold drop in titre shown by most of the sensitive viruses can certainly not be explained in such a way.

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## The Investigation of Influenza and Related Viruses in the Electron Microscope, by a New Technique

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**SUMMARY:** Influenza and related viruses were studied by a new method in which virus is adsorbed on the membranes of laked fowl red cells for examination in the electron microscope. The numbers of virus particles adsorbed per unit area of red-cell membrane were estimated from direct counts in micrographs of palladium-shadowed and unshadowed specimens. There was a definite saturation level of adsorption for each strain of virus, the value varying also according to the particular batch of cells and their age after storage at 0°. For subsaturation conditions the number of particles adsorbed was proportional to the concentration of virus and the concentration of cells, and a function both of time of contact and temperature. The relationships were complicated by the fact that elution began before adsorption was complete.

The comparative data for the size and variation of these viruses were obtained from micrographs of preparations directly adsorbed from infective allantoic fluid and thus not subjected to harsh methods of purification. The mean sizes ( $m\mu$ ) found for the dried virus were: influenza A virus,  $90 \pm 11.5$ ; influenza B virus,  $103 \pm 8$ ; fowl-plague virus,  $101 \pm 16$ ; Newcastle-disease virus,  $193 \pm 28$ ; and mumps virus,  $179 \pm 28$ .

The occurrence of long forms associated with influenza B virus was confirmed and filamentous structures were demonstrated for the first time in preparations of a strain of fowl-plague virus.

The red-cell agglutination first described by Hirst (1941, 1942) and McClelland & Hare (1941) for influenza virus and fowl erythrocytes has proved of great value as a basis for the assay of influenza and related viruses. Attempts to elucidate the process of this agglutination have given some information on the mechanism of attack of a virus on a susceptible cell, particularly on the initial phases of adsorption and penetration. An excellent survey of the lines of approach so far taken in this vital problem has been recently given by Burnet (1948). Our own interest in this field developed during investigations of the physical properties of the influenza, Newcastle disease, fowl plague and mumps group of viruses (Elford, Chu, Dawson, Dudgeon, Fulton & Smiles, 1948), where the haemagglutination test was extensively applied, and it was thought that the process would be more clearly understood if direct optical evidence of the mechanism were obtained. The sizes of the viruses, 70–150  $m\mu$ , lie on both sides of the limit of resolution of the ultra-violet light microscope. In some ultra-violet micrographs of the virus of Newcastle disease, taken in collaboration with our colleagues, Mr J. Smiles and Mr F. W. Welch, some adsorbed bodies were demonstrable in 0.1  $\mu$  optical sections taken through the cell; but the limitations of the method were such that no convincing evidence was forthcoming. The electron microscope appeared to be the obvious tool for the study, the resolution being more than adequate; and the sole requirement for its use being a suitable method of preparing the specimen.

We were interested in the haemagglutination phenomenon for another reason.

In our early electron microscope studies of the morphology of the viruses of this group we had to examine material which had been purified by prolonged centrifugation at high speeds. The effects of such strong gravitational fields on virus morphology were quite unknown, but it was suspected that a considerable amount of distortion could take place. Although we had a reasonably good correlation between infectivity and the numbers of particles seen on the micrograph, we were uncertain whether all the material was virus, or whether in fact it had contained associated material in the same size range as the virus. This was particularly the case with Newcastle-disease virus, where purified preparations showed particles of similar size but of widely differing structure. The variability of Newcastle-disease virus preparations has been noted by other workers (Cunha, Weil, Beard, Taylor, Sharp & Beard, 1947; Bang, 1946, 1947, 1948). There is another objection to the isolation of viruses by centrifugation; it yields a limited range of particle sizes for study. Successful isolation depends on the ability to select particles of a given size range from materials of a wide range of size and density. The technique may have serious limitations when evidence of the mode of virus multiplication is required. Were the process one of simple binary fission the narrow range of size of the particles isolated would be sufficient to contain all the relevant virus particles, and the loss of larger particles during centrifugation would be immaterial. There is, however, no real reason to suppose that this is the case. Should the multiplication be characterized by forms greatly above or below the modal size then losses during centrifugation may well be responsible for our failure to detect essential links in the chain of development. Adsorption to red cells, on the other hand, seemed ideal for our purpose, being presumably independent of size and density of the particles, the two properties predominantly made use of in previous methods of purification.

The intact red cell is too thick for electron microscopy by ordinary transmission methods; and examination of its surface structure by the replica technique is beset by as yet unsolved difficulties. However, Wolpers (1941) has shown that the ghosts of human erythrocytes lysed osmotically can be successfully photographed in the electron microscope, with or without fixation by osmic acid. Likewise, Dawson & McFarlane (unpublished) found the laked avian erythrocyte to possess ideal thickness and contrast properties for electron microscopy. For example, in the photograph of a shadowcast preparation in Pl. 1, fig. 1 the folds and also a considerable degree of fine structure in the membrane are well shown. The membrane is approximately 6  $m\mu$  thick and is therefore quite thin enough to act as a support for virus particles in transmission, as distinct from replica, studies of virus adsorption. Influenza virus adsorbed on such laked cell membranes gave a clear picture of the virus bodies (Dawson & Elford, 1949), and we have now made similar studies of all the viruses in this group. During the course of the work Heinmets (1948) published some micrographs of purified influenza virus adsorbed on the avian and human erythrocytes. He was concerned solely to demonstrate the adsorption and examined only purified virus material; no data on the quantitative aspects of the adsorption phenomenon were reported.

## METHODS AND MATERIALS

Fowl red cells were haemolysed with saponin according to the method of Dounce & Lan (1948) and the laked cells washed thoroughly with 0.9 % saline m/33 phosphate pH 6.7. After a given period of contact with virus at 0° the cells were centrifuged and the deposit, resuspended in 0.9 % saline, fixed in 0.1 % osmic acid and washed with distilled water several times by spinning at 500 r.p.m. for 5 min. and discarding the supernatant fluid. The cells were then mounted directly from the distilled water suspension on ordinary electron microscope disks with supporting collodion film. As the suspension settles on the film the concentration of cells is checked under the optical microscope, and adjusted by pipetting off the excess suspension or adding more of the original material until there are about six cells deposited in each field of 0.01 mm.<sup>2</sup>. The specimen is then examined by direct transmission or after shadow-casting with gold or palladium. Where the laked cells are in high concentration there is considerable folding of the membrane during the drying. For counts of particles on the membrane it is always possible by careful mounting to obtain flat uniform fields with minimum folding. The area to which the count relates is measured on the photographic plate by a planimeter and the number of virus particles per square micron ( $\mu^2$ ) of the cell surface determined.

*Strains of viruses.* The following viruses were tested.

The PR8 strain of influenza A and the Lee strain of influenza B. Each has been passaged in 10-day eggs and harvested on the twelfth day.

Newcastle disease—'Herts' strain kindly supplied by Dr T. M. Doyle.

Fowl plague—'Dutch' strain also supplied by Dr T. M. Doyle.

Newcastle-disease virus and fowl-plague virus were each passaged in the allantois in 10-day hen's eggs and the virus harvested after 36 hr. incubation at 37°.

Mumps—Enders EMA 41 strain kindly sent to us by Dr H. Koprowski. This virus was passaged in the allantois in 8-day eggs and harvested after 4 days' incubation at 35°.

*Virus assay.* The red-cell agglutination was carried out according to Salk (1944). The limiting infective dilution (L.I.D.) was determined by inoculating serial 10-fold dilutions of the virus into eggs of the appropriate age (see Beveridge & Burnet, 1946).

*Concentrations of cells.* Red and laked cell suspensions were counted in a haemocytometer cell.

*Abbreviations.* [virus] and [cells]=concentration of virus and of cells respectively.

## RESULTS

*The adsorption of influenza viruses*

Influenza virus was readily adsorbed from the unpurified allantoic or amniotic fluid of infected hens' eggs. Pl. 2, fig. 8 and Pl. 4, fig. 9, show cell membranes on which influenza B virus has been adsorbed, and Pl. 1, fig. 2 and Pl. 4, fig. 8 the corresponding micrographs of influenza A. The virus particles occur singly

and in groups in the membrane surface and are not distributed in any obvious pattern. There is very little interference caused by adsorbed non-viral allantoic material, which is relatively small both in size and quantity. No particles of the size and shape of influenza virus were seen on cells exposed to allantoic fluid from normal eggs; and chemical analysis for protein confirmed that the amount of adsorbed material was small. With concentrated preparations of influenza virus it was possible to pack the surface of the membrane with virus particles as illustrated in Pl. 2, fig. 4. The packing arrangement gives no obvious indication of any geometric pattern of receptor groups on the membrane surface. Admittedly the arrangement is complicated by the fact that we observe simultaneously the particles which lie on the upper surface of the specimen and also those underneath, which are covered by two thicknesses of cell membrane. The results do indicate, however, that adsorption is the outcome of a direct interaction between each virus particle and the cell membrane, and is not a process whereby the initial adsorption of a few virus particles provides foci for the aggregation of more virus particles in that area; that is, a process analogous to ordinary chemical crystallization. Virus adsorbed at 0° may be eluted by incubating at 37°. Such eluted virus can be reabsorbed on to fresh membranes. Chemical analysis and the examination of a series of micrographs both show that there is less associated non-viral protein on the cell membrane after adsorption of eluted virus. Influenza virus inactivated for 30 min. at 56° is readily adsorbed, but cannot subsequently be eluted by incubation; this confirms the findings of Hirst (1942). There were no morphological differences between influenza A virus particles adsorbed directly from allantoic fluid, or after heat-inactivation; or reabsorbed after elution.

Virus was adsorbed on to intact red cells which were then haemolysed with saponin. The membranes were quite free of virus; in the laking process the virus had apparently been eluted. This result emphasized the need of very thorough washing of the laked cells in order to remove traces of saponin which could inhibit subsequent adsorption. Influenza virus was successfully adsorbed in the range of maximum stability, pH 5.5–7.6. We found that both virus and cells could be stored at 0° for several weeks without serious deterioration, as judged from subsequent micrographs.

*The adsorption of the viruses of Newcastle disease, fowl  
plague and mumps*

Our earlier studies of these viruses (Elford *et al.* 1948) were made with centrifugally purified preparations. Elementary particles of the virus of fowl plague were relatively uniform in size and shape, but those of mumps and Newcastle-disease viruses were variable both in size and morphology. The correct interpretation of this apparent pleomorphism has hitherto been uncertain. Does it represent a variable virus structure, or is it largely induced by excessive manipulation of the virus during purification? Investigation of the effects of various electrolyte concentrations on the dispersion of Newcastle-disease virus by Bang (1947, 1948) and our own observations on purified

concentrates of the virus (Elford *et al.* 1948) led to the conclusion that much of the variability in form was due to deleterious treatment of the virus. The laked-cell adsorption technique gives further evidence. The mean sizes of all the viruses in the group (Table 1) and the scatter of the sizes (Fig. 1) were determined. In each instance 200 virus particles were measured from micrographs of unshadowed specimens. When particles were oval in outline the larger

Table 1. *Comparative data on the sizes of the viruses of the influenza, Newcastle disease, fowl plague and mumps group, in dried preparations*

| Virus             | Strain              | General morphology   | Mean size and standard deviation (m $\mu$ ) |
|-------------------|---------------------|--|---|
| Influenza A       | P.R. 8              | Round bodies with sharp outline                                | 90 $\pm$ 11.5                               |
| Influenza B       | Lec                 | Round bodies with sharp outline                                | 103 $\pm$ 8                                 |
|                   |                     | Associated long forms  | 110<br>(up to 4 $\mu$ long)                 |
| Fowl plague       | Dutch               | Round bodies with rather diffuse outline; apparently flattened | 101 $\pm$ 16                                |
|                   |                     | Associated long forms  | 80 width<br>(up to 6 $\mu$ long)            |
| Newcastle disease | Herts               | Round and oval bodies with sharp outline                       | 193 $\pm$ 28                                |
| Mumps             | Enders<br>E.M.A. 41 | Round and oval bodies; appreciably flattened                   | 179 $\pm$ 28                                |

dimension was measured. The measurement (microscope calibration) is accurate to within  $\pm 5\%$  of the true value. It is noteworthy that the figures are for virus fixed with osmic acid. Whether air-dried, osmic-acid fixed material, or air-dried unfixed material more nearly represents the virus in its normal state is possibly rather an academic question. The shrinkage occurring when fixed material is dried is generally more symmetrical than is the case with unfixed material. The measurements in Table 1 and Fig. 1 are reliable because they are made on preparations subjected to the minimum amount of manipulation.

With mumps (Pl. 3, fig. 7) and Newcastle-disease (Pl. 3, fig. 6) viruses there is indeed a rather wide variation in the size of adsorbed particles. Mumps virus dries very flat on the membrane with an overall size range, 100–260 m $\mu$ , and a mean of 179  $\pm$  28 m $\mu$  (Fig. 1). Newcastle-disease virus dried in a variable manner. Sometimes the shadowed image indicated a round flat body and at others an ovoid shape. The range of particle size was 140–270 m $\mu$  with the mean 193  $\pm$  28 m $\mu$ .

The whole question of morphological variation among viruses must clearly be reconsidered in the light of the striking micrographs of fowl-plague and influenza B viruses directly adsorbed from freshly harvested allantoic fluid. In addition to the normal round bodies of fowl-plague virus 101  $\pm$  16 m $\mu$  in diameter (Pl. 3, fig. 5), there are filaments up to 6  $\mu$  in length and averaging 80 m $\mu$  in width, which in some instances are terminated by a spherical mass 100 m $\mu$  in diameter. These filaments would be lost during filtration and spinning

and we have been able to detect them only by the adsorption method. Pl. 2, fig. 8 and Pl. 4, fig. 10 show the analogous but rather less-striking result with influenza B virus. Long forms in influenza-infected allantoic fluid have already been reported by Mosley & Wyckoff (1946) for the PR8 and Weiss strains of influenza A and the Lec strain of influenza B. The morphological and developmental significance of these anomalous forms can be elucidated only by examination of virus material in various stages of development. Up to the

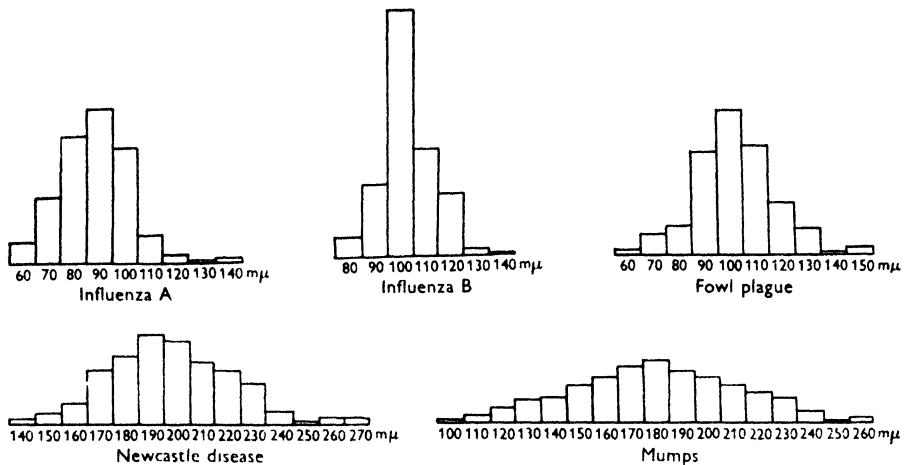


Fig. 1. The distribution of particle sizes in preparations of influenza and related viruses. The figures refer to the mean size value in mμ for each rectangle in the histograms.

present no such filamentous structures have been encountered in normal allantoic fluid and they are not associated in any way with the original red-cell preparation. The fact that long forms are, like the round particles, adsorbed to the red cell suggests that both have a similar surface structure. Furthermore, these long forms are not a feature only of aged and partially degenerated virus, since all our observations were made on fresh material. We have also succeeded in eluting both long and round forms from adsorbed heat-treated fowl-plague virus, as happens with influenza virus, by treatment with specific antiserum. This constitutes additional evidence of a similarity in the surface structure of the round and long forms.

#### *The factors influencing adsorption*

**Virus concentration.** A constant number of laked red cells was added to a given volume of each of three suspensions of influenza virus at different concentrations. After 15 min. contact at 0° the cells were centrifuged and the virus adsorbed on the membranes counted. Table 2 gives the results of such an experiment with influenza B virus (see also Pl. 4, figs. 9–11).

Clearly the specific adsorption is directly proportional to the concentration of virus for the prevailing conditions, and this relationship can be expected to hold so long as the ratio [cells] to [virus] is such that full saturation of the membrane surface is not approached.



*Cell concentration.* The results with a wider range of cell and virus concentrations are given in Table 3, which records experiments all done with one batch of red cells. With the concentrated virus, prepared by adsorption from allantoic fluid on to laked cells at 0°, followed by elution at 37° in a reduced

Table 2. *The effect of virus concentration on the number of influenza B particles adsorbed per unit area of red-cell membrane, in a suspension of laked red cells ( $5 \times 10^8$ /ml.)*

| Dilution of virus (eluate) | Virus haemagglutinin titre | Area counted ( $\mu^2$ ) | Particle count | Particles/ $\mu^2$ |
|----------------------------|----------------------------|--------------------------|----------------|--------------------|
| 10 <sup>0</sup>            | 12800                      | 40.7                     | 910            | 22                 |
| 10 <sup>-1</sup>           | 1280                       | 71.5                     | 139            | 1.9                |
| 10 <sup>-2</sup>           | 128                        | 64.6                     | 13             | 0.2                |

Table 3. *The effect of variation in concentration of influenza B virus and laked cells on the number of virus particles adsorbed per unit area of red-cell membrane*

| Exp. no. | Haemagglutinin titre of virus | Laked cells (million/ml.) | Particle count | Area counted ( $\mu^2$ ) | Particles/ $\mu^2$ | Percentage decrease in haemagglutinin titre after adsorption |
|----------|-------------------------------|---------------------------|----------------|--------------------------|--------------------|--|
| 1        | 32000                         | 12                        | 844            | 28                       | 30                 | 90-99  |
| 2        | 32000                         | 3                         | 723            | 25                       | 29                 | < 50   |
| 3        | 32000                         | 0.75                      | 580            | 16.4                     | 35                 | < 50   |
| 4        | 3200                          | 12                        | 110            | 28                       | 4                  | 90   |
| 5        | 3200                          | 3                         | 340            | 25                       | 14                 | < 50   |
| 6        | 3200                          | 0.75                      | 543            | 21                       | 26                 | < 50   |
| 7        | 3200                          | 3                         | 468            | 21                       | 22                 | —  |
| 8        | 3200                          | 3                         | 476            | 13.5                     | 35                 | --   |

Exps. 1-7, contact 15 min. at 0°; Exp. 8, 15 min. at 37°.

In Exps. 7 and 8 the virus was heated 30 min. at 55.5°.

volume of 0.9 % saline-phosphate pH 6.7, the number of particles adsorbed per unit area, within the error of measurement, was the same for each [cell]; i.e. the adsorption capacity of the receptors on the cell surface had been saturated in each instance. When the [virus] was diminished 10-fold, saturation was approached only for the lowest [cells]. Several experiments have yielded a definite saturation value which varies widely with different batches of cells.

*Heat treatment of virus.* Virus heated for 30 min. at 55.5° was adsorbed, under otherwise similar conditions, to a greater extent than was normal virus (Table 3, Exps. 5 and 7). The increase was even more pronounced when the heat-treated virus was adsorbed at 37° (Exp. 8). We have already noted that heated virus is not readily eluted; so that the dynamics of its adsorption differ from those of normal virus. Since the time of contact between virus and cells in our experiments was constant throughout, the results indicate that in the absence of spontaneous elution the rate of adsorption increases with increase in temperature. The improved adsorption of heat-treated virus was also manifest when three successive amounts of virus were adsorbed on the same cells (Table 4).

There is clearly an increment in the number of particles adsorbed for each successive treatment in subsaturation conditions. Similar experiments with normal fresh virus gave rather inconsistent figures. In some cases there was no apparent increase in the count of adsorbed virus, and in others there was a slight rise. The complicating factor of spontaneous elution superimposed on the effect of different [virus]: [cells] ratios probably accounted for the variable results, and their evaluation would demand far more extensive studies.

Table 4. *The effect of successive adsorptions of heat-treated influenza B virus upon the same red-cell membrane, on the number of virus particles adsorbed per unit area*

| No. of adsorption | Particle count | Area counted ( $\mu^2$ ) | Particles/ $\mu^2$ |
|-------------------|----------------|--------------------------|--------------------|
| 1                 | 124            | 25                       | 5                  |
| 2                 | 110            | 12                       | 9                  |
| 3                 | 136            | 8                        | 17                 |

[Virus]; haemagglutinin titre 1600 initially, 800 after heating.

[Cells] =  $3 \times 10^6$ /ml. Contact = 15 min. at  $0^\circ$ .

Table 5. *The effect of temperature on the degree of adsorption of influenza B virus per unit area of laked red-cell membrane*

| Temperature ( $^\circ$ C.) | Particle count | Area counted ( $\mu^2$ ) | Particles $\mu^2$ |
|----------------------------|----------------|--------------------------|-------------------|
| 0                          | 310            | 19                       | 16.5              |
| 20                         | 288            | 15                       | 19                |
| 37                         | 270            | 25.5                     | 10.5              |

[Virus]; haemagglutinin titre = 1600.

[Cells] =  $1.5 \times 10^6$ /ml. Contact = 15 min.

*The effect of temperature.* The effect of temperature on the degree of adsorption of normal influenza B virus from allantoic fluid was also investigated (Table 5).

The figures are consistent with the view that there are two opposing processes with positive though different temperature coefficients. The rate of elution at  $0^\circ$  is relatively small; it increases much more rapidly with temperature than does the rate of adsorption, appearing to overtake it in the region of  $20^\circ$ , where the number of particles remaining adsorbed after 15 min. is maximum.

*The influence of time; the adsorption isotherm.* Mixtures of virus and laked cells, kept at  $0^\circ$  with periodic shaking, were sampled at intervals and the adsorption curves for each of the viruses was determined for periods up to 2 hr. In some instances preliminary observations on the rate of elution were made.

(i) *Influenza virus.* Our preliminary results with two strains of influenza virus confirmed the findings of Hirst (1942). The curves for influenza A and influenza B are given in Fig. 2*a*. The rate of adsorption of the B strain was such that no significant increase in virus concentrations on the cell membrane was noted after 5 min. With the A strain, on the other hand, adsorption did not approach completion until after 100 min.

(ii) *Fowl-plague virus*. The particle count/unit area of membrane reached a steady high value after 10 min., suggesting that saturation had been reached for the prevailing conditions (Fig. 2*b*).

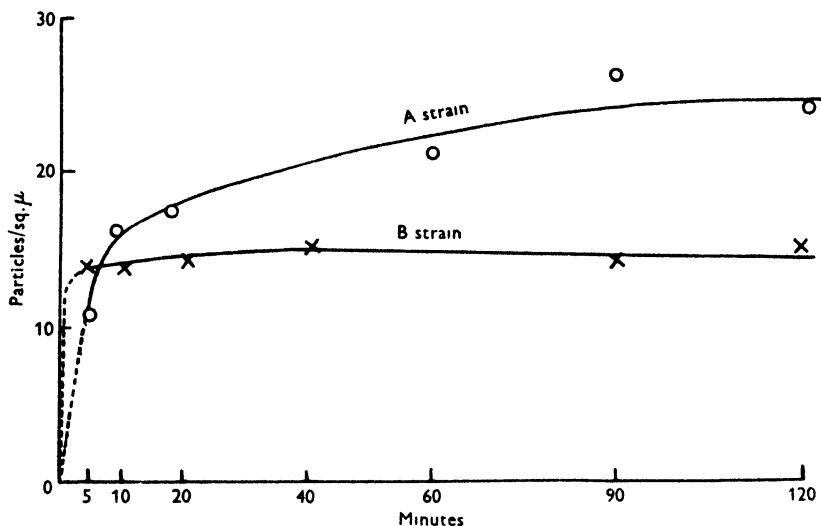


Fig. 2(a). The adsorption isotherm for influenza virus strains A and B.

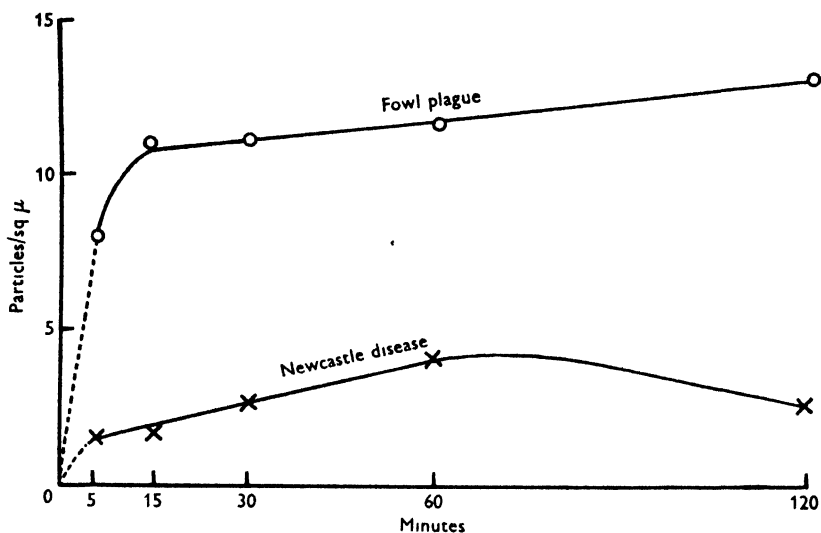


Fig. 2(b). Adsorption isotherm for Newcastle-disease virus and for fowl-plague virus.

(iii) *Newcastle-disease virus*. The adsorption in this case was a maximum after 60 min. and then decreased to the extent of nearly 50 % during the next hour (Fig. 2*b*).

(iv) *Mumps virus*. Our observations for mumps virus indicate that here, too, the adsorption is slow.

The adsorption curves are for freshly harvested unheated virus, and therefore

portray the combined effect of adsorption and elution at 0°. The form of the curve for Newcastle-disease virus suggests that the rate of elution is in this case appreciable even at this low temperature.

*Elution of virus.* Our observations on the elution of the individual viruses indicate that whereas influenza B virus is eluted to the extent of 90 % or more in 1 hr. at 37°, Newcastle-disease and fowl-plague viruses are much more tenacious, 25 % still remaining on the membrane after 1 hr. Accepting the current view that elution is determined by enzymic action as a result of which the receptor linkage is destroyed, we can explain these findings by assuming that a proportion of the virus particles are without enzyme, or, that the enzyme varies in its specificity and stability from virus to virus. A further point arising from our elution experiments concerns the appearance of the membrane after the elution of virus. No alteration could be detected. We conclude that the receptor groups are relatively small elements in discrete areas of the cell membrane.

### DISCUSSION

The adsorption method of preparing viruses for electron microscopy has three outstanding advantages. (1) It is simple and saves much time and effort in preparing specimens. Viruses that ordinarily require 2–3 hr. high speed centrifugation for their sedimentation, can, when adsorbed on the red-cell membranes, be deposited in 5 min. at low speeds. The specimens may thus be quickly washed several times and mounted without any harsh treatment. (2) The specificity of the adsorption means that viruses, in freshly harvested infected fluids, are quickly freed from protein and other products. The possibility thus afforded of examining the virus at quite short intervals after the initiation of infection is being exploited in the hope of obtaining new evidence of the morphology of viruses during the most active phase of multiplication. (3) Direct counts of the virus particles actually adsorbed on the membranes can be used to explore certain cell-virus relationships under varied conditions. The virus is well distributed on the membranes and there is no tendency to the aggregation that frequently mars preparations made by drying directly on a collodion film.

The method is limited to viruses that can be adsorbed on the red-cell membrane. We are, however, studying the dissociated haemagglutinins, as obtained with vaccinia virus, with promising results. The structure of the membrane itself is also under investigation, and already some direct transmission micrographs of the osmic-acid fixed membrane have given evidence of a strongly electron-adsorbing material distributed in the form of a relatively open network within the membranes.

A full understanding of the interaction of laked red cell and virus demands more data concerning the spontaneous elution of virus, but the facts so far established clearly indicate the extent to which the net adsorption is dependent upon virus concentration, cell concentration, time and temperature. The membrane appears to possess a definite specific adsorption capacity. This saturation level varies with the particular batch of cells and their age, i.e. time

of storage at 0° after preparation. For freshly made suspensions the observed average saturation values in experiments with influenza B was close to  $40/\mu^2$ , with a variation of as much as  $\pm 20$ . The mean particle diameter for influenza B virus was  $103\text{ m}\mu$ . Hence the area covered by forty such particles is  $0.33\mu^2$  indicating that 67% of the membrane surface remains uncovered. Hanig (1948), working with PR8 influenza A and using haemagglutinin titres for deducing the adsorption on intact red cells, calculated that only 1/80th of the surface was covered at saturation, the number of virus particles/cell being 298. The saturation level of adsorption in Exp. 1, Table 3, with influenza B was 30 per  $\mu^2$  with a [cells] =  $12 \times 10^6/\text{ml}$ . The total surface area of a laked fowl red cell from electron micrographs is  $1.8 \times 10^{-6}\text{ sq.cm.}$ , and the average number of virus particles adsorbed/cell is calculated to be  $5.4 \times 10^3$ . Therefore the total virus adsorbed from 1 ml. suspension was  $6.5 \times 10^{10}$ , and this from the haemagglutination test represented 90–99% of the virus. Unfortunately, we had not determined the limiting infective dilution of this virus suspension. Clearly here is a means of determining the approximate number of virus particles/ml. We found it possible to pack considerably more particles on the surface by using a sufficiently concentrated suspension of virus. The lack of any geometric arrangement in such close packing suggests that the excess virus may be held non-specifically by aggregation rather than by attachment to residual receptor groups. Alternatively, the force of attraction between the receptors and virus might vary because the receptor groups are not all equally accessible; for example, they might be distributed in more than one plane.

A knowledge of the adsorption and elution curves for the viruses is essential for devising optimum conditions of purification. We have already pointed out the advantages of using laked cell suspensions instead of intact red cells (Elford *et al.* 1948). Furthermore, the data of adsorption and elution may form a useful basis for characterizing viruses within the group; even strains of influenza viruses A and B behave quite differently.

The observations that will probably arouse great interest are those revealing the long forms associated with fowl-plague and influenza B viruses. The existence of the filamentous forms does not alter the fact that the round elementary particle is the morphological unit, capable, when injected into the host, of multiplying and giving rise to the typical symptoms of the particular disease. It would be premature to regard these long forms as being a phase in virus multiplication, but their close association with the elementary virus bodies in freshly harvested infective fluids and the similarity of their surfaces with regard to combination with red cells or antibody are suggestive. Inspection of the lower membrane in Pl. 2, fig. 3 reveals both circular bodies of influenza B virus,  $100\text{ m}\mu$  in diameter, and rod-like forms up to  $750\text{ m}\mu$  long. We are trying to determine whether or not these rods represent a stage in the development of the still larger forms up to  $2\mu$  long that are occasionally seen.

The ratio of the sizes of the largest to the smallest forms of the elementary virus bodies is much the same for all members of this group, namely 2 to 2.5:1. The scatter of sizes is characteristic for each virus preparation studied. Thus with the viruses of influenza A and B and of fowl plague (Fig. 1) there is

a definite peak at the mean-size value, whereas with Newcastle-disease virus and particularly with mumps virus the distribution is more even. It is instructive to consider the general character of the dried virus particle on the membrane. The elementary bodies of influenza A and B yielded sharply defined images with appreciable shadows indicating a height:base ratio ( $h:b$ ) of the order 0.25 and 0.8 respectively; and for fowl-plague virus a ratio of *c.* 0.25. Newcastle-disease virus was more variable, with a greater proportion of flattened types, the  $h:b$  being *c.* 0.125–0.2. Mumps virus appeared as a flat circular body with an  $h:b$  of *c.* 0.1. The distribution of sizes and the structure and mode of drying of the virus particle are clearly related. Information is needed about the manner in which a particle dries in relation to its structure, density, surface properties, interfacial tension with the supporting surface, and to the medium and conditions of drying. That dried virus particles may yield vastly different images according to their previous treatment is especially clear from our studies of Newcastle-disease and mumps virus purified by four to six centrifugal washings in dilute electrolyte and then mounted and dried on collodion film. The elementary bodies of mumps virus had a mean size  $140 \pm 30$  m $\mu$  with an  $h:b$  of *c.* 0.3. The disparity between this and our present figure is doubtless due to factors modifying the process of drying.

Our figures for the sizes and distribution of the elementary particles of influenza viruses A and B are in good agreement with those of Sharp, Taylor, McLean, Beard & Beard (1944) (see also Beard, 1948) and Lauffer & Stanley (1944). The strain of Newcastle-disease virus we have studied is different from the strains reported upon by American workers (Cunha *et al.* 1947; Bang, 1946, 1947, 1948), but our latest observations support our earlier views of the morphology of the virus (Elford *et al.* 1948). We found none of the gross pleomorphism evident in micrographs of centrifuged concentrates of the virus. Our data for mumps virus agree well with those of Weil, Beard, Sharp & Beard (1948) for the same strain. To our knowledge no electron-microscope studies on fowl-plague virus, other than our own, have as yet been published and the value  $101 \pm 16$  m $\mu$  for the mean size of this virus adsorbed on the red-cell membrane accords well with the value  $83 \pm 15$  m $\mu$  we obtained earlier from measurements on purified virus.

The investigation of these viruses by the new technique has generally substantiated the conclusions reached in earlier work and greatly clarifies the morphology of these viruses. At the same time it has confirmed the existence of filamentous forms of influenza B virus and demonstrated for the first time similar structure in preparations of fowl-plague virus.

The purified preparations of Newcastle-disease, fowl-plague and mumps viruses examined in our earlier work, we are now convinced, consisted essentially of virus without any large proportion of associated material. Nevertheless, in some instances they may not have been representative of the virus in its original state, in that certain filamentous structures were inadvertently discarded during the purification.

We gratefully acknowledge the help received from our colleagues Drs C. H. Andrewes, F. Fulton and T. H. Flewett in passaging the viruses and conducting

infectivity tests from time to time. We also thank Dr A. Isaacs for his co-operation in the experiments demonstrating the displacement of influenza virus from the membrane by specific antibody.

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1

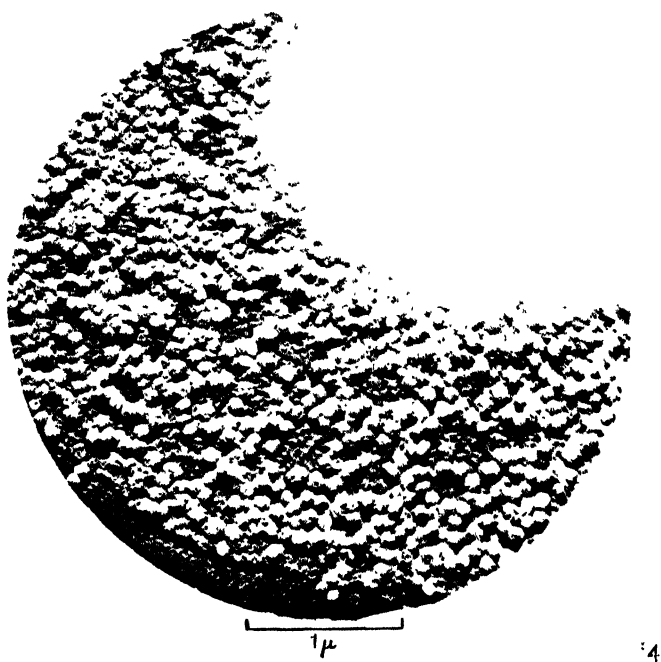


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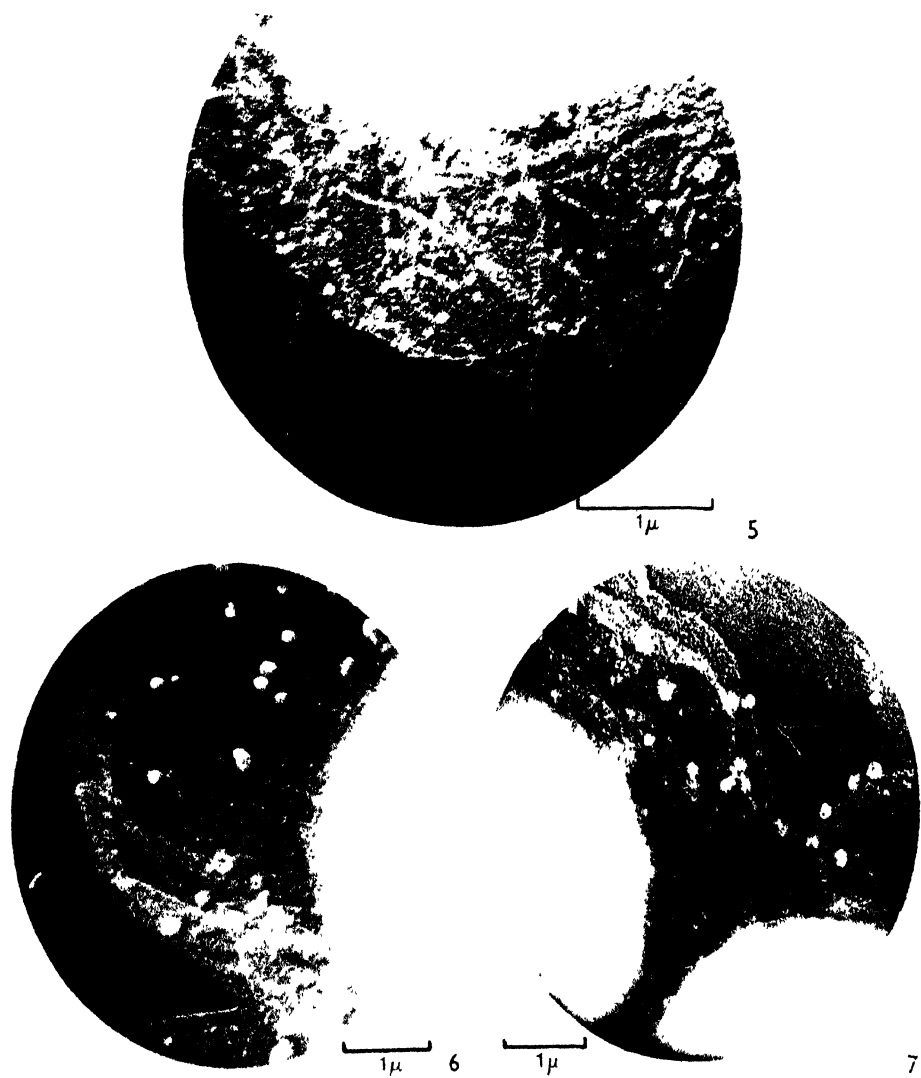
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Figs. 1-2

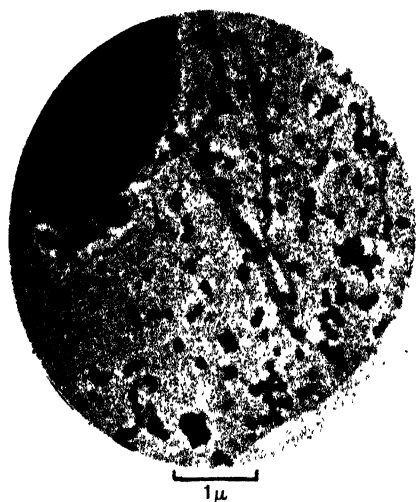




Figs. 3-4



Figs. 5-7



8



9



10



11

Figs. 8-11

EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Laked fowl erythrocyte shadowcast with palladium. The cell nucleus appears as an oval white body to the right of the field. The cell membrane is folded.
- Fig. 2. Laked fowl erythrocyte with adsorbed influenza A virus, shadowcast with palladium. The virus particles appear as small disks on the membrane surface.

PLATE 2

- Fig. 3. Transmission micrograph of laked fowl erythrocytes with adsorbed influenza B virus.
- Fig. 4. Laked fowl erythrocyte with a high concentration of adsorbed influenza B virus to illustrate the random arrangement of virus on close packing. Preparation shadowcast with palladium.

PLATE 3

- Fig. 5. Laked fowl erythrocyte with adsorbed fowl-plague virus, showing the round elementary bodies and associated long forms. Preparation shadowcast with palladium.
- Fig. 6. Laked fowl erythrocyte with adsorbed virus of Newcastle disease. Preparation shadowcast with palladium.
- Fig. 7. Laked fowl erythrocyte with adsorbed mumps virus. Preparation shadowcast with palladium.

PLATE 4

- Fig. 8. Transmission micrograph of laked fowl erythrocyte with adsorbed influenza A (PR8) virus.
- Figs. 9, 10 and 11. Laked fowl erythrocyte membranes with successive 1:10 dilutions of influenza B virus adsorbed on the membrane. Fig. 10 shows two of the characteristic long forms associated with influenza B virus. These forms are readily distinguishable from folds in the cell membrane also visible in this micrograph. All preparations shadowcast with palladium.

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## A Technique for Examining Large Numbers of Bacterial Culture Filtrates by Partition Chromatography

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**SUMMARY:** A description is given of apparatus and methods which have been used to produce large numbers of satisfactory and reproducible chromatograms of bacterial culture filtrates. Some of the factors which influence the preparation and development of chromatograms are discussed.

While studying the nitrogen metabolism of micro-organisms by means of paper chromatography (Woiwod & Proom, 1948; Proom & Woiwod, 1949*a, b*); Linggood & Woiwod, 1948), it was necessary to prepare large numbers of single-dimensional paper chromatograms. During this work some of the conditions for obtaining reproducible and satisfactory chromatograms were determined. As little detailed descriptive work on the technique of paper chromatography has been published since the original observations of Consden, Gordon & Martin (1944), description of the apparatus and methods employed in this laboratory is given in the hope that it will prove of use to workers wishing to use this elegant analytical method.

In partition chromatography on paper an organic solvent such as butanol, saturated with water, is allowed to pass over a dried spot of a solution of the material under investigation placed on a strip of filter-paper. The process is carried out in a closed chamber in an atmosphere saturated with water and solvent. The various constituents present in the spot are separated by this process and on completion of the process will have travelled different distances from the starting line. The distance will depend on the distribution coefficient of the substance between water and the organic solvent used and on other physico-chemical factors, some of which are not fully understood. After drying, the paper is examined under ultra-violet light and then sprayed with the appropriate reagent to reveal the positions of the separated constituents of the original spot. In the case of amino-acids and peptides, the reagent is ninhydrin, which produces coloured spots on heating. The ratio of the distance the substance has run to the distance the solvent front has travelled is known as the  $R_f$  value and varies with the solvent employed. It is a useful means of indicating the approximate position which a substance will occupy on a chromatogram. It cannot be used by itself to identify an amino-acid or peptide with certainty; this can be done only by noting other things in addition, such as shade of colour produced by the ninhydrin, position relative to other amino-acids,  $R_f$  value in some second solvent and by running on the same chromatogram a control mixture containing known amino-acids.

## APPARATUS AND METHODS

**Tanks and Accessories.** Rectangular tanks 2 ft. long, 1 ft. wide and 3 ft. deep, which can be opened at the top, and are capable of accommodating up to eight sheets of chromatograms on filter-paper  $22\frac{1}{2}$  in. by  $18\frac{1}{4}$  in., are used. De-scaled quality stainless steel sheet, c. 0.06 in. thick, is the most satisfactory material.

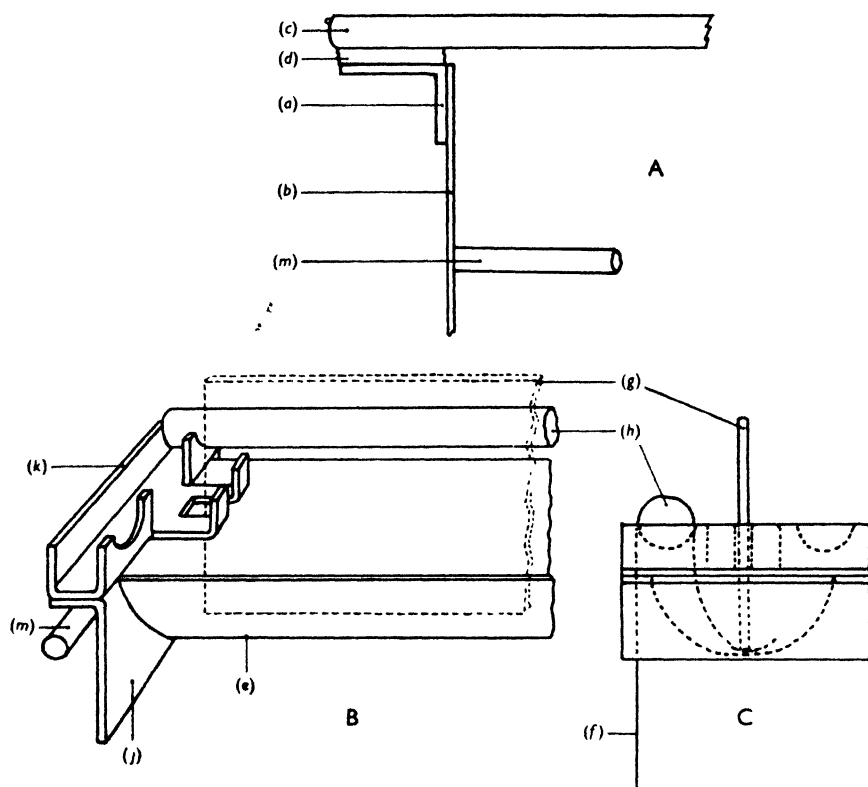


Fig. 1. A, method of sealing tank; B, side view of trough; C, end view of trough.

Welded tanks of this material withstand prolonged exposure to *n*-butanol + acetic acid mixture, phenol and 'collidine', with no sign of corrosion. The method of closing the top of the tank to form a solvent-tight seal is shown in Fig. 1 A. A welded angle-iron frame (a) is soft soldered to the top of the tank (b) which is closed by a sheet of plate glass (c) resting on a sponge-rubber gasket (d) fixed to the frame with Bostik C compound (B.B. Chemical Co. Ltd., Leicester). Removable troughs fitting inside the tank hold the water-saturated solvent. De-scaled stainless steel sheet is satisfactory for the troughs and is more robust than glass. A simple design (Fig. 1 B, C) has enabled easily cleaned troughs of standard size to be produced. The body of the trough (e),  $22\frac{3}{4}$  in. long, is semicircular in cross-section ( $1\frac{1}{2}$  in. diam.). One edge of the filter-paper sheet (f) dips into the trough and is held in position by a removable stainless

steel plate (*g*). The rest of the paper, after passing over a glass support (*h*), hangs vertically in the tank. The end-closure (*j*) and the support (*k*) which holds the glass rods and stainless steel plate, are welded on to the end of the trough, which is supported in the tank on  $\frac{1}{4}$  in. diameter stainless rod (*m*) fixed  $1\frac{3}{4}$  in. below the top.

**Paper and solvents.** When single-dimensional chromatograms are being used to study the changes produced by micro-organisms in a complex medium such as an acid-hydrolysate of casein, paper and solvent must be so chosen as to give the best possible separation of the amino-acids on the paper. At present no better combination has been found than Whatman No. 4 paper ( $22\frac{1}{2}$  by  $18\frac{1}{4}$  in.) with *n*-butanol + acetic acid mixture as solvent (Partridge, 1948). Adequate separation is obtained overnight (16 hr.), the solvent front usually passing off the paper in this time. For two-dimensional chromatography phenol is used as solvent for the second run at right angles to the first. Leucine cannot be separated from *iso*-leucine nor valine from methionine with this combination.

*n*-Butanol is redistilled before use and only the fraction distilling between 116 and 118° is used. The *n*-butanol + acetic acid mixture is made by adding 30 ml. of glacial acetic acid (Analar) to 250 ml. of a 50/50 (v/v) mixture of *n*-butanol and water, in a graduated 250 ml. cylinder. After shaking, the mixture rapidly separates into two layers, the bottom one having a volume of 90 ml. Any marked variation from this volume indicates that something is wrong, usually that the butanol is impure or the acetic acid below strength. The amount of acetic acid added is fairly critical. The bottom aqueous layer (aqueous acetic acid saturated with butanol), is separated and placed in a beaker in the bottom of the tank. This should be changed for every run. The top, butanol, layer (butanol saturated with aqueous acetic acid), is used in the trough for running the chromatogram. It is not satisfactory to use this solvent again as ester formation takes place; only sufficient should be made for the number of chromatograms to be run at one time.

Any good-grade phenol is usually satisfactory, and a trace of colour does not seem to affect the separation of the amino-acids or to produce an unsatisfactory solvent front. It was found convenient to buy phenol in 500 g. amounts and to liquefy this with 225 ml. water. On solution and shaking, an emulsion forms which often needs a day to clear; phenol saturated with water is the heavier phase; this is used in the trough. When a rapid separation of the emulsion is obtained, the material is likely to prove unsatisfactory. A beaker of water saturated with phenol is placed in the bottom of the tank; this need be changed only at long intervals.

When using phenol or *n*-butanol + acetic acid mixture a beaker of water is also placed in the tank as this aids the attainment of equilibrium conditions. Any phenol solution remaining in a trough after a run can be returned to a stock bottle and used again after resaturating with water. 'Collidine', a crude product consisting mainly of trimethylpyridines, has also been used. This material needs purification before use and because of its unpleasant penetrating odour, its greater sensitivity to changes of temperature than either *n*-butanol +

acetic acid mixture or phenol, and the difficulty in eluting material from chromatograms run with it is for this reason not favoured in this laboratory.

*Temperature.* Because the solvents used are saturated with water at a definite temperature, any large temperature variations during a run may cause the appearance of a second phase consisting of water saturated with solvent, or the solvent may not remain saturated. In either case unsatisfactory chromatograms are produced. As already mentioned, 'collidine' is particularly sensitive to such changes; a rise of less than  $1^{\circ}$  will cause cloudiness in a water-saturated 'collidine' solution. Phenol and the *n*-butanol + acetic acid mixture are very much less affected by temperature changes and will tolerate variations of  $\pm 2^{\circ}$  about a mean without the chromatograms being seriously affected. Chromatograms employing these last two solvents can be run between  $15$  and  $30^{\circ}$  without much effect on the separation obtainable, the higher temperatures giving faster running chromatograms. The problem of temperature control therefore resolves itself, with these two solvents, into holding tank temperatures steady to within  $\pm 2^{\circ}$  of any temperature between  $15$  and  $30^{\circ}$  for a period of about 16 hr. This was possible under all but the most exceptional climatic conditions by keeping the tanks in an ordinary room with all windows closed and heating the room electrically, the electric heaters being thermostatically controlled and of sufficient capacity to deal with the rapid drop in temperature sometimes experienced at night at certain times of the year. The tanks should stand well away from the windows.

Both phenol and *n*-butanol + acetic acid mixture run off the paper in 16 hr. Often this does not matter, but it may be necessary at times to study material moving very close to the solvent front. By adjusting the amount of solvent in the trough it is possible to regulate the distance the solvent front travels; 25 ml. is sufficient to allow the solvent front to remain on the paper when the chromatogram is run along the  $22\frac{1}{2}$  in. length of a sheet of No. 4 Whatman paper. The paper should, however, be removed from the tank as soon as possible after the front has become stationary; otherwise the spots lose their sharpness because of diffusion.

*Preparation of paper.* When comparisons have to be made of single-dimensional chromatograms of culture-filtrates run for various periods, some standardization of spot size and, therefore, of volume of material put on the paper at the starting line, is necessary. This may be done without difficulty by a very simple procedure. Chromatograms are normally run in the long direction of the paper. A base line is drawn at right angles to the  $22\frac{1}{2}$  in. edge. The distance of this line from the top of the paper is such that the spots will be clear of the glass supporting-rod and on the side away from the trough when the paper is in position. Positions for spots are marked along this line, starting 1 in. from the side and with  $\frac{3}{4}$  in. between spots. At 0.2 in. from this line and parallel to it a second line is drawn. A short length of capillary tubing drawn to a fine point is filled by dipping its tip into the culture-filtrate to be examined. The tip of the capillary is then applied to the paper at one of the marked spots and removed when the circumference of the circle of liquid formed reaches the second line. With this technique, spots with a volume of about 0.004 ml. and



of standard size can be placed on the paper. If a solution is considered to be too weak to give a satisfactory chromatogram, more spots are added at the same site, each spot being allowed to dry before each new addition.

With two-dimensional chromatograms a similar technique is employed but the spot size is decreased to a diameter of 0.1 in., since more satisfactory chromatograms are obtained with this smaller spot. No hard and fast rules can be made about the amount of material needed to obtain a chromatogram of satisfactory strength. As a rough guide, however, it was found that one spot of a culture medium containing about 8  $\mu$ g. amino-nitrogen will give an excellent single-dimensional picture with *n*-butanol-acetic acid mixture as solvent. Ninhydrin colours are weaker after two-dimensional running and the spots tend to be less sharply defined. Between 50 and 100  $\mu$ g. amino-nitrogen were found necessary to give satisfactory two-dimensional pictures with the same culture filtrate.

*Drying of papers.* After a solvent-run papers are dried by hanging them in a steam-heated chamber. This was constructed from a bacteriological steamer approximately 3 ft. long, 2 ft. 6 in. wide and 2 ft. deep standing on one end so that the 'lid' now forms a vertical door (3 ft. high by 2 ft. 6 in. wide) hinged at one side. The chamber is heated internally by steam pipes and is lagged externally. The internal temperature attained is about 80°. The door is made fume-proof by a rubber gasket, and can be tightly closed by means of two bolts with butterfly nuts at top and bottom. A small centrifugal blower feeds air into the bottom of the chamber and the fumes pass through a 2 in. stainless steel pipe in the top of the chamber, led outside the building. With this apparatus a dozen papers can be dried with almost no escape of fumes into the room other than those arising when the papers are transferred from the tanks to the dryer.

After running the chromatogram the troughs with the papers are removed from the tank. The papers are suspended by 'bulldog' clips from the bottom edge in the hot chamber; to prevent tearing, a strip of dry filter-paper is used to strengthen this edge before applying the clips. Drying is then continued until the amino-acid and peptide spots examined under ultra-violet light show a reasonably strong fluorescence. At 70°–80° the drying usually takes from 2 to 3 hr. The examination under ultra-violet light, besides indicating when a paper is ready for spraying, may give other useful information; for instance, it is possible to detect flavins (Crammer, 1948; Woizwod & Linggood, 1948), porphyrins (Rimington, 1948), and fluorescent bacterial pigments.

*Colour development.* A solution of ninhydrin in chloroform (0.1 %, w/v), with 0.1 % 'collidine' added, is the most satisfactory reagent for spraying the papers. No other solvents so far tested produce such strong colours. The reason for this is not clear, but it is possible that chloroform modifies the complex reaction taking place between amino-acids and peptides and the ninhydrin. Dried sheets are removed to a fume-cupboard and sprayed, the operator wearing a respirator. The paper is returned to the heating chamber and the developed spots examined after about 15 min., the heating being continued for another hour to ensure maximal development of colour. The small percentage of 'collidine' aids colour differentiation and is particularly useful in distinguishing glycine and

serine when using the *n*-butanol + acetic acid mixture. Both these amino-acids have very similar  $R_f$  values in this solvent and produce very similar colours with ninhydrin in chloroform. In the presence of a trace of 'collidine', however, serine produces a blue coloration and glycine is dull red. It has thus been found possible to follow on single-dimensional chromatograms the disappearance of one or the other acid from a medium containing both.

The use of chloroform raises problems when large numbers of sheets have to be sprayed. Unless the spraying can be done in an efficient fume chamber and the operator works in a respirator, it is probably more satisfactory to use *n*-butanol as a solvent for the ninhydrin. The spray should not be too fine, as the amount of ninhydrin on the paper before heating has a marked effect on colour development, and subsequent respraying and heating will not intensify a chromatogram. A volume of 75 ml. is needed to spray adequately a sheet 22½ by 18½ in. This is achieved by two sprayings, a procedure advisable with chloroform as it dries so rapidly that portions of the chromatogram can be missed in the first spraying.

*Recording of results.* The most satisfactory records are photographs, a written note being made of those points which, because of shades of colour or for other reasons, will not be obvious on the print. Colour photography has proved disappointing and is not recommended.

#### General observations

The achievement of stable conditions of saturation in the tanks is of prime importance in obtaining satisfactory chromatograms. To this end it is most important not to leave tanks open longer than is necessary when removing troughs and papers. For the same reason it is not advisable to use any given tank for more than one solvent; it often takes a day or more to regain equilibrium after changing solvents. Unsatisfactory results may also be obtained when first using a new tank, but after once coming to equilibrium with a given solvent there is no further difficulty.

With the techniques described it is possible to run ten culture-filtrates and two controls of uninoculated medium in duplicate on one sheet, using the 18½ in. edge as starting line. As each tank holds three troughs and will if necessary hold four, up to 200 single-dimensional chromatograms can be run each night in one tank.

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## The Examination, by Partition Paper Chromatography, of the Nitrogen Metabolism of Bacteria

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**SUMMARY:** Some 300 strains of bacteria representing twenty genera were grown on an acid-hydrolysed casein medium. Using the paper chromatographic technique of Woiwod (1949) a preliminary survey was made of the amino-acid and polypeptide composition of the bacterial culture filtrates. It was proved that: (a) changes may occur in the filtrate chromatogram which have group or species significance; (b) bacteria with simple nutrient requirements, i.e. which utilize ammonia, do not affect the chromatogram in the initial growth stage; (c) whenever the chromatogram is affected serine is the first amino-acid to be metabolized; (d) Gram-positive bacteria eliminate the aspartic acid spot but leave the basic amino-acid group unaffected, and the reverse occurs with Gram-negative bacteria; (e) many bacteria synthesize ninhydrin-positive material, presumably polypeptide, the synthesis of a given kind of polypeptide being sometimes associated with a particular group or species of bacteria. By a disintegrator technique it was demonstrated that bacteria store many free amino-acids inside the cell.

The application of partition paper chromatography to the qualitative analysis of complex mixtures of amino-acids and polypeptides by Consden, Gordon & Martin (1944) has a general appeal because it is an attractive and simple method which has no parallel in the classical methods of protein analysis. Within the limitations of the method it is possible, in a reasonable time, to obtain a general picture of some of the aspects of the nitrogen metabolism of bacteria grown in a selected medium; and also to examine a sufficient number of cultures to distinguish strain, species and group differences. We have made a general survey of the amino-acid and polypeptide metabolism of some 300 strains of bacteria, representing twenty genera, grown on a casein hydrolysate medium. The purpose of this paper is to describe the general picture and the problems which have arisen out of this survey. The more detailed examination of selected problems will be the subject of further studies.

### METHODS

**Medium.** Bacteria were grown in a liquid medium consisting of an acid-hydrolysate of casein (3 g. N/l.), yeast extract, growth factors and metallic salts with 0.1 % added glucose. The medium was arbitrarily selected with the object of giving clear chromatograms uncomplicated by the initial presence of polypeptide or protein. The amount of glucose was kept to a minimum in order to prevent pH changes due to acid production during growth. The growth factors, yeast extract and metallic salts were added in an attempt to extend the range of bacteria which would grow in the medium. The exact composition and preparation of the medium is as follows.

Casein acid-hydrolysate  $\equiv$  3 g. N/l.

Yeast extract, 100 ml.

Glucose, 1.0 g.

NaCl  $\equiv$  0.5 %

Tryptophan 0.2 g.

MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.45 g.

CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.01 g.

ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.008 g.

MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.008 g.

Riboflavin, 200  $\mu$ g.

Calcium pantothenate, 400  $\mu$ g.

Biotin, 4  $\mu$ g.

Aneurin, 200  $\mu$ g.

Uracil, 15 mg.

Adenine, 15 mg.

Pyridoxin HCl, 1.2 mg.

*p*-Aminobenzoic acid, 40  $\mu$ g.

Nicotinic acid, 600  $\mu$ g.

Choline chloride, 7.5 mg.

Pteroylglutamic acid, 0.2  $\mu$ g.

Distilled water to 1 l.

The casein hydrolysate was prepared by hydrolysing Glaxo light white soluble casein with *c.* 6N hydrochloric acid. Total nitrogen determinations were made on the concentrated digest in order that the nitrogen content of the final medium could be adjusted to 3 g./l. The yeast extract was prepared by boiling 500 g. bakers' yeast with 2 l. of distilled water for 30 min. and filtering through paper. The growth factors and metallic salts were added as convenient volumes of stock solutions. The medium was made up to the required volume with distilled water and the pH adjusted to 7.6. The medium was heated to 95°, sterilized by filtration through a Berkefeld-type candle and filled aseptically into suitable containers which were then incubated to test for sterility.

*Growth.* The medium was dispensed in 20 ml. lots in 1 oz. screw-capped bottles, and where necessary anaerobiosis was established by the addition of 0.01 % thiolacetic acid. The medium was seeded with a loopful of culture from the appropriate solid medium or with a few drops of a suitable liquid culture. The cultures were incubated at 37° for 3 days and then were divided into two parts. A 5 ml. portion was removed into a sterile screw-capped bottle and incubated for a further 7 days. To the remainder were added a few drops of bromocresol purple and sterile N-HCl until a yellow colour developed. Acetate buffer (3 ml. of M/25) was added to give a final pH of 4.5 and the culture incubated for a further 7 days at 37°. These periods were selected arbitrarily, on the basis of preliminary experiments, to distinguish changes that take place in the medium during the active growth stage and changes developing after growth has almost ceased. In this medium during the growth of any of the organisms examined the reaction was either unaltered or alkaline (pH 7.6–9.0), so that the effects observed were those taking place at slightly alkaline reaction. In order to examine effects due to enzyme activity at acid reaction, part of the culture was adjusted to the arbitrarily selected pH of 4.5, as described above. Unless otherwise stated in the text, cultures were always grown in this way.

*Chromatographic analysis.* Culture filtrates were examined by Woiwod's (1949) technique. The solvent used for one-dimensional paper chromatography was *n*-butanol+acetic acid mixture; phenol was used as the second solvent for two-dimensional work. In each case the stationary phase was water. The chromatograms were run on sheets of Whatman No. 4 paper (22½ × 18½ in.). Each filtrate was tested in duplicate and ten filtrates were examined on each

sheet of paper. At each end of the series of spots on the paper the uninoculated medium was always included as a control. The chromatograms, after development, were examined, the results noted and a permanent photographic record taken. Pl. 1, fig. 1, shows a one-dimensional chromatogram of the control medium, with a key showing the position of the various amino-acids present in the casein digest.

## RESULTS

### *Removal of amino-acids during the first three days of growth*

The chromatographic picture of the filtrates from a culture after 3 days' growth may differ in a number of ways from that of the control medium. The effects are illustrated in Pl. 1, fig. 2. With some organisms, such as *Pseudomonas aeruginosa* and *Bacillus subtilis*, the chromatogram is unaffected in spite of heavy growth. With our technique no definite conclusions can be drawn about the removal of amino-acids from the medium for the direct building of bacterial proteins, since in most of the growths obtained the nitrogen of the cells did not represent more than 3 % of the total nitrogen initially present in the medium. With *Ps. aeruginosa*, however, during the first 3 days an opacity equivalent to  $15 \times 10^9$  organisms/ml., or 0.3 g. N/l., was obtained by shaking flask cultures. This amount of nitrogen corresponded to about 10 % of that initially present in the medium, and had it been taken exclusively from the amino-acid constituents it would almost certainly have been visible on the chromatogram.

With bacteria requiring more complex nutrients, i.e. specific amino-acids and other essential metabolites, a change in the chromatogram always takes place during the early growth. This is illustrated in Pl. 1, fig. 2 with *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Shigella dysenteriae* (Shiga), *Escherichia coli*, *Pasteurella bovisseptica*, *Serratia marcescens* and *Proteus vulgaris*. In all cases the serine spot decreases in intensity, and in many the glycine spot is unaffected. This is also illustrated in Pl. 1, fig. 3, which shows the decrease in serine with ten strains of *Shigella paradysenteriae* (Boyd types; *Sh. boyd*). This plate also shows that the effect is clearly defined and occurs to an equal extent with a number of different strains. During this survey we confined our attention to changes of this order, which are not only unequivocal but are not subject to strain variation. With many Gram-negative organisms the aspartic acid spot increases in intensity; in Pl. 1, fig. 2, the increase with *Esch. coli* and *Ser. marcescens* is very evident. This increase proved, in some cases at least, to be due to the formation of polypeptides.

A number of Gram-negative species metabolize the basic amino-acids. This is illustrated in Pl. 1, fig. 2, by *Ser. marcescens*. *Vibrio comma* does so markedly, the basic amino-acid group often being completely eliminated within 2 days. With *Pr. vulgaris* the valine and leucine groups are attacked; with other organisms these groups are not usually metabolized during the first 3 days. With many Gram-positive organisms the aspartic acid spot disappears from the chromatogram (Pl. 1, fig. 2, with *Str. pyogenes*, *Staph. aureus* and *Coryne. diphtheriae*). A list of the groups of organisms tested and the amino-acids removed during the first 3 days' growth is given in Table 1. In most cases

at least ten strains were examined in each group. Differences between species within a group were also observed, and the use of more suitable media is expected to reveal many others. For example, in medium enriched with soluble starch *Haemophilus paraptussis*, but not *H. pertussis*, attacked the leucine and valine groups.

Table 1. *The effect of growth of organisms of different genera on the main amino-acid spots as they appear on the single-dimensional chromatogram*

| Period of incubation ... | 3 days at alkaline reaction |          |        |          |         |        |         | 10 days at alkaline reaction |          |        |          |         |        |         | 3 days at alkaline and 7 days at acid reaction |          |        |          |         |        |         |
|--------------------------|-----------------------------|----------|--------|----------|---------|--------|---------|------------------------------|----------|--------|----------|---------|--------|---------|--|----------|--------|----------|---------|--------|---------|
|                          | Basics                      | Aspartic | Serine | Glutamic | Alanine | Valine | Leucine | Basics                       | Aspartic | Serine | Glutamic | Alanine | Valine | Leucine | Basics   | Aspartic | Serine | Glutamic | Alanine | Valine | Leucine |
| Genus                    |                             |          |        |          |         |        |         |                              |          |        |          |         |        |         |  |          |        |          |         |        |         |
| <i>Pseudomonas</i>       | -                           | -        | -      | -        | -       | -      | -       | +                            | +        | +      | +        | +       | +      | +       | +  | +        | +      | +        | +       | +      | +       |
| <i>Vibrio</i>            | +                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | x       | -      | -       | +  | -        | +      | +        | +       | -      | -       |
| <i>Neisseria</i>         | -                           | -        | +      | -        | -       | -      | -       | x                            | -        | +      | +        | -       | -      | -       | +  | -        | +      | +        | -       | -      | -       |
| <i>Alkaligenes</i>       | -                           | -        | -      | -        | -       | -      | -       | x                            | -        | +      | +        | -       | -      | -       | +  | -        | +      | +        | -       | -      | -       |
| <i>Escherichia</i>       | x                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | x       | -      | -       | +  | +        | +      | +        | +       | +      | +       |
| <i>Aerobacter</i>        | x                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | -       | -      | -       | +  | +        | +      | +        | +       | +      | +       |
| <i>Klebsiella</i>        | +                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | -       | -      | -       | +  | -        | +      | +        | +       | +      | +       |
| <i>Serratia</i>          | +                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | x       | -      | -       | +  | -        | +      | +        | +       | +      | +       |
| <i>Proteus</i>           | -                           | -        | +      | -        | -       | +      | +       | +                            | +        | +      | +        | +       | +      | +       | +  | +        | +      | +        | +       | +      | +       |
| <i>Salmonella</i>        | x                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | -       | -      | -       | +  | -        | +      | +        | x       | +      | +       |
| <i>Shigella</i>          | -                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | -       | -      | -       | +  | -        | +      | +        | +       | -      | -       |
| <i>Pasteurella</i>       | -                           | -        | +      | -        | -       | -      | -       | +                            | -        | +      | +        | -       | -      | -       | -  | -        | +      | +        | +       | -      | -       |
| <i>Haemophilus</i>       | -                           | -        | +      | -        | -       | x      | x       | +                            | -        | +      | +        | -       | +      | +       | +  | -        | +      | +        | +       | x      | x       |
| <i>Bacillus</i>          | -                           | -        | -      | -        | -       | -      | -       | -                            | +        | +      | +        | +       | +      | +       | x  | +        | +      | +        | +       | +      | +       |
| <i>Mycobacterium</i>     | -                           | -        | -      | -        | -       | -      | -       | -                            | +        | +      | +        | -       | -      | -       | -  | +        | +      | +        | -       | -      | -       |
| <i>Staphylococcus</i>    | -                           | +        | +      | -        | x       | -      | -       | -                            | +        | +      | +        | +       | -      | -       | -  | +        | +      | +        | -       | -      | -       |
| <i>Diplococcus</i>       | -                           | +        | +      | -        | -       | -      | -       | -                            | +        | +      | +        | +       | -      | -       | -  | +        | +      | +        | +       | -      | -       |
| <i>Streptococcus</i>     | -                           | +        | +      | -        | -       | -      | -       | -                            | +        | +      | +        | x       | -      | -       | -  | +        | +      | +        | x       | -      | -       |
| <i>Lactobacillus</i>     | -                           | +        | +      | -        | -       | -      | -       | -                            | +        | +      | +        | x       | -      | -       | -  | +        | +      | +        | x       | -      | -       |
| <i>Corynebacterium</i>   | -                           | x        | +      | -        | -       | -      | -       | -                            | +        | +      | +        | -       | -      | -       | -  | +        | +      | +        | -       | -      | -       |
| <i>Clostridium</i>       | -                           | x        | +      | +        | -       | -      | -       | -                            | +        | +      | +        | -       | -      | -       | -  | +        | +      | +        | -       | -      | -       |

+ = Marked decrease of amino-acid with all strains.

x = Slight decrease of amino-acid with all strains.

or x = Decrease with some strains.

- = No effect.

#### *Removal of amino-acids during prolonged incubation at acid or alkaline reaction*

After prolonged incubation at 37°, at either acid or alkaline reaction, a further series of changes may occur in the chromatogram. The changes are usually more marked at the acid reaction and may be so extensive that all ninhydrin-positive material is removed from the chromatogram. From the summary of the results in Table 1, it is clear that changes occurring during the first 3 days usually progress to near or complete extinction of the corresponding amino-acid spot. Organisms, such as *B. subtilis* or *Ps. aeruginosa*, that have simple nutrient requirements and which initially cause little change in a chromatogram, may produce most extensive changes on further incubation. There is a sharply

defined difference between Gram-positive and Gram-negative organisms. The former do not attack the basic amino-acid group but remove aspartic acid early, whereas the latter produces the reverse effect.

A series of chromatograms of the filtrates of bacterial cultures taken at intervals gives an overall picture of the succession of changes taking place in the medium—a picture that can hardly be obtained by any other method. Pl. 2, fig. 2, shows the effect of incubation at acid reaction on a number of cultures of *Esch. coli*. The following changes can be seen: elimination of serine and decrease in basic amino-acid group (strain 2); elimination of alanine, glutamic acid and the basic amino-acid group (strains 4, 7, 1, 3); appearance of polypeptide in the aspartic acid position (strains 5, 7, 9); appearance of polypeptide near the tyrosine position (strains 1, 3–9); decrease in the valine group and the leucine group with polypeptides in the aspartic acid and tyrosine positions (strain 6); finally, almost complete elimination of ninhydrin-positive material from the chromatogram, only traces of the two polypeptides being left (strain 8). If samples from strain 8, for example, had been taken at intervals during incubation the changes would have occurred in the above order. It should be noted that in Pl. 2, fig. 2, there is a drift in the chromatogram, due to irregularities during the solvent-run, which on casual examination might lead to misinterpretation. Thus with strain 4 the glutamic acid spot might appear to be in the alanine position; however, closer examination of the chromatogram reveals the nature and extent of the drift.

#### *Possible synthesis of polypeptides*

During the examination of the filtrates from some species a number of ninhydrin spots appeared on the chromatogram which were not present in the basal medium. Since they did not correspond in position, and in some cases in ninhydrin colour, to any of the amino-acids present in the basal medium, we suggest that they are likely to be polypeptides. In the case of *Cl. tetani* one such ninhydrin-positive spot which appeared on the chromatogram below the leucine group (Pl. 2, fig. 1 (3)), was eluted, acid-hydrolysed and shown by two-dimensional chromatography to be a polypeptide. In other cases the polypeptide nature of these spots has not yet been unequivocally demonstrated. These presumed polypeptides were often different for different species since they appeared in various positions on the chromatogram. The effect is illustrated in Pl. 2, fig. 1. They may appear during the early growth stage, as with *Pr. vulgaris* and *Cl. tetani*, or later after growth has ceased, as with *Esch. coli* and *Sh. dysenteriae*. They may appear at alkaline reaction or only at acid reaction and their appearance is always associated with the disappearance of a particular amino-acid from the chromatogram. This associated amino-acid may be different for different species; for example, it is glutamic acid with *Cl. tetani* (Pl. 2, fig. 1 (3)), leucine group with *Pr. vulgaris* (Pl. 2, fig. 1 (5)), alanine with *Staph. aureus* and proline with *Cl. bifermentans* (Pl. 2, fig. 1 (1)). The association between the amino-acids and the production of 'polypeptides' is evident, for when the particular amino-acid was not removed no 'polypeptide' appeared



and, moreover, the strength of the 'polypeptide' spot varied inversely with the strength of the amino-acid spot. This inverse relationship is clearly shown in Pl. 2, fig. 2, with nine strains of *Esch. coli*. The 'polypeptide' spot, which appears just above the tyrosine position on the chromatogram, is absent from the control medium, very intense with strains 1, 3 and 5 when the glutamic acid has disappeared, and just visible with strains 2 and 4 where the glutamic acid is relatively unaffected.

Table 2. *Synthesis of polypeptides by bacteria, showing the position of the polypeptide spot on the chromatogram, the amino-acid which is associated with the production of the polypeptide and the reaction at which the polypeptide is formed*

| Genus or species   | Position of polypeptide on chromatogram                         | Associated amino-acids       | Reaction of filtrate |
|--|---|------------------------------|----------------------|
| <i>Aerobacter</i> , <i>Escherichia</i> ,<br><i>Salmonella</i> , <i>Serratia</i> ,<br><i>Shigella</i> | On aspartic acid spot   | Serine                       | Alkaline             |
| <i>Escherichia</i> , <i>Shigella</i><br><i>Proteus</i>   | On tyrosine spot<br>Two polypeptides after<br>leucine group     | Glutamic<br>Leucine<br>group | Acid<br>Alkaline     |
| <i>Staphylococcus</i><br><i>Cl. tetani</i>   | Before tyrosine spot<br>After leucine group                     | Alanine<br>Glutamic          | Alkaline<br>Alkaline |
| <i>Cl. bifermens</i><br><i>Cl. sordellii</i>   | Two polypeptides one<br>before tyrosine, one<br>on valine group | Proline                      | Alkaline             |

The hydrolysis of the *Cl. tetani* polypeptide showed that it was not a polymer of the associated glutamic acid nor had it an exceptionally high glutamic acid content. Table 2 lists the 'polypeptides' so far observed which give intense spots on the chromatogram. In addition to these, many indefinite spots suggesting 'polypeptide' formation have been observed; further examination with more favourable media is likely to show many others.

#### *Amino-acids and polypeptides inside the cell*

Using a tissue disintegrator (supplied by H. Mickle, Romeyn Works, Romeyn Road, London, S.W. 16) and shaking a dense suspension of washed organisms with small glass beads (Ballotini Grade 9), as described by Elliot & Gale (1948), the storage of ninhydrin-positive material inside the cell is being examined. Some of the preliminary results are shown in Pl. 2, fig. 3. It can be seen that many amino-acids are stored within the cells. The basic amino-acid group, aspartic acid, serine, glutamic acid, the valine and leucine groups are clearly visible in the chromatogram of disintegrated *Staph. aureus*. With the exception of glutamic acid these are also visible in the disintegrate of *Esch. coli*. There is little evidence of the presence of polypeptides.

#### *Hydrolysis of whole bacteria*

The organisms *H. pertussis*, *Pr. vulgaris*, *Aerobacter aerogenes*, *Coryne. hoffmanni*, *Myc. phlei*, *Diplococcus pneumoniae*, *Esch. coli*, *B. subtilis*, *Past. bovisseptica* and an unidentified chromogenic coccus, were grown on the appro-

priate medium and the cells removed and washed free from the medium by six successive washings with physiological saline of centrifuged deposits. The organisms were hydrolysed by refluxing for 40 hr. with 6 N-HCl. The hydrolysate was evaporated to dryness *in vacuo* and redissolved in a little distilled water. Single- and two-dimensional chromatograms showed the presence of the following amino-acids and groups of acids, which were present in all cases: cystine, aspartic acid, glutamic acid, serine, glycine, threonine, lysine, histidine, arginine, alanine, proline, the leucine group, the valine group, and tyrosine. No evidence of previously unknown amino-acids was obtained in any of the chromatograms. Although it is always possible that such acids may be present in bacteria, it is fairly clear that they could not be present in amounts greater than 2 % of the total amino-acids. No gross differences between the amino-acid compositions of different species of bacteria were evident with this technique.

### DISCUSSION

Partition paper chromatography makes possible a new approach to the study of the nitrogen metabolism of bacteria. This survey shows that it is possible to obtain a fairly complete picture of the amino-acid and polypeptide composition of bacterial filtrates, and to make comparative studies of large numbers of strains or species with great facility. Moreover, under reasonably standard conditions, the results are reproducible and many of the effects unequivocal.

It is, however, necessary to point out two limitations of our method which affect the interpretation of the results. First, in spite of the extreme sensitivity of the ninhydrin reaction, many of the changes which take place in bacterial cultures, such as the utilization of specific amino-acids as essential nutrients, occur at a level which is below the sensitivity of the method. Secondly, although the chromatogram records the presence or absence of ninhydrin-positive material it throws little direct light on the mechanism of the reactions which may be taking place.

There appears to be a general relationship between the nutrient requirements of an organism and the effect of growth on the amino-acid composition of the medium. Usually those organisms with simple nutrient requirements (capable of using ammonia) do not affect the chromatogram during the initial growth stage. This is illustrated in Pl. 1, fig. 2, with *B. subtilis* and *Ps. aeruginosa*.

The results with *Ps. aeruginosa* would suggest that in a mixture of ammonium salts and amino-acids organisms which can utilize the ammonium salts do so in preference to amino-acids. It is one of the advantages of the chromatographic method that the effects obtained in complex mixtures can be studied. It is also of interest to note that organisms with simple nutrient requirements often produce the most extensive changes in the chromatogram on prolonged incubation at alkaline or acid reaction.

With organisms having more complex nutrient requirements a change in the chromatogram always takes place during growth. The outstanding observation here is the universal utilization of serine. The preferential use of serine suggests that the amino-acid plays a prominent part in bacterial metabolism. The

mechanism of the reaction and the reasons for the selection of this particular amino-acid will be studied further.

Besides serine, other amino-acids may be metabolized during and after the initial growth. Both species and group differences are apparent. Perhaps the most interesting is the difference between Gram-positive and Gram-negative organisms, illustrating once again the profound dissimilarity of these two groups of bacteria.

The synthesis of polypeptides during and after growth, the mechanism of their production and the function of the associated amino-acids, are of special interest. Polypeptide synthesis by bacteria has often been regarded as a rather unusual aspect of bacterial metabolism, little being recorded except the formation of some polypeptide antibiotics and the capsular substance of *B. anthracis*. It seems, however, that polypeptide synthesis may be a more general phenomenon in bacterial metabolism than hitherto recognized. Whether the ninhydrin-positive materials responsible for the unidentified spots are in all cases polypeptides remains to be seen. But it is clear that these ninhydrin-positive materials exhibit all grades of specificity, varying from the group specificity shown by the 'polypeptide' appearing in the aspartic acid position, which is synthesized by most Gram-negative bacteria and the two 'polypeptides' produced by all members of the genus *Proteus*, to the species-specific polypeptide of *C. tetani*. Both in the removal of amino-acid and in the synthesis of 'polypeptide' group and species differences are apparent. These differences are likely to be of value in bacterial classification.

Using specific decarboxylase preparations Gale (1947) has shown that bacteria store a number of free amino-acids inside the cell. Preliminary observations with the chromatographic method confirm this, and show that other amino-acids are also stored. Taylor's (1947) observation that Gram-positive, but not Gram-negative, organisms store glutamic acid is also confirmed (Pl. 2, fig. 3).

Contrary to Polson's (1948) observations, the amino-acid composition of hydrolysates of twelve species of bacteria examined by two-dimensional chromatography did not include any previously unknown amino-acids.

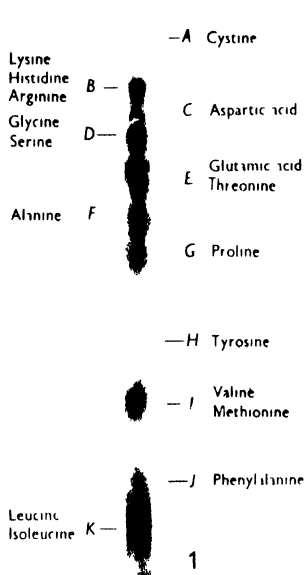


Fig. 1



Fig. 3



Fig. 2

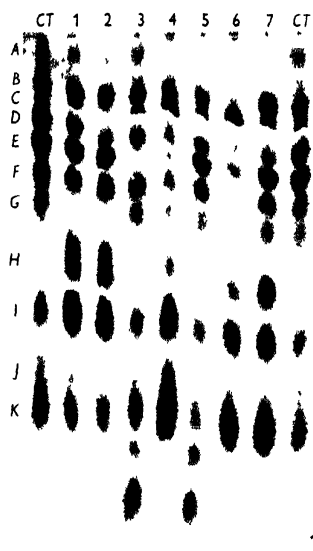


Fig. 1

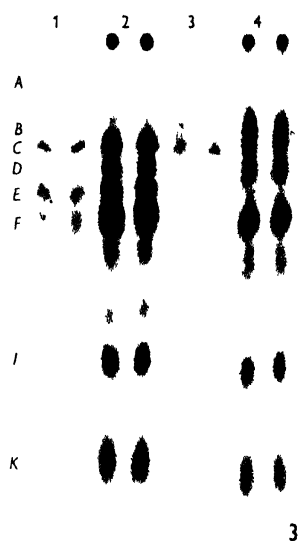


Fig. 3

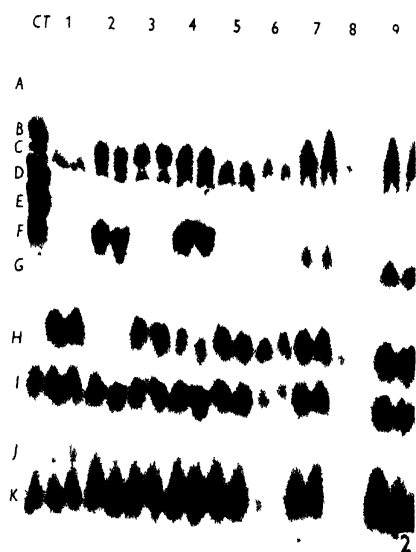


Fig. 2

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## EXPLANATION OF PLATES

## PLATE 1

- Fig. 1. Key chromatogram of control medium showing the positions of the amino-acids on the chromatogram. The capital letters on the left of the chromatograms refer to this key.
- Fig. 2. Chromatogram of 3-day filtrates from bacterial cultures: CT=Control medium (i.e. uninoculated). 1, *B. subtilis* (no change); 2, *Str. pyogenes* (decrease in aspartic acid and serine); 3, *Staph. aureus* (decrease in aspartic acid and serine and appearance of 'polypeptide'); 4, *Coryne. diphtheriae* (decrease in aspartic acid and serine); 5, *Ps. aeruginosa* (no change); 6, *Sh. dysenteriae* (decrease in serine); 7, *Ser. marcescens* (decrease in serine, basic group, increase in aspartic acid); 8, *Pr. vulgaris* (decrease in serine, leucine and valine groups, appearance of 'polypeptide'); 9, *Esch. coli* (decrease in serine, increase in aspartic acid); 10, *Past. boydii* (decrease in serine).

Fig. 3. Chromatogram of 3-day filtrates from cultures of ten strains of *Sh. paradysenteriae* (Boyd types) showing decrease in serine and consistency of the effect. CT=Control medium; 1-10=filtrates.

## PLATE 2

- Fig. 1. Chromatogram of filtrates of cultures showing 'polypeptide' formation. CT=control medium (i.e. uninoculated). 1, *Cl. bifementans* ('polypeptide' above tyrosine and on valine group); 2, *Cl. sordellii* ('polypeptide' above tyrosine and on valine group); 3, *Cl. tetani* ('polypeptide' below leucine group); 4, *Ser. marcescens* ('polypeptide' in aspartic acid position); 5, *Pr. vulgaris* ('polypeptide' below leucine group); 6, *Esch. coli* at alkaline reaction ('polypeptide' in aspartic acid position); 7, *Sh. paradysenteriae* ('polypeptide' just above tyrosine spot). CT=control medium (i.e. uninoculated). Associated amino-acids: 1, 2, proline; 3, glutamic acid; 4, ? serine; 5, leucine and valine groups; 6, ? serine; 7, glutamic acid.
- Fig. 2. Chromatogram of 10-day filtrates at acid reaction of nine strains of *Esch. coli* showing appearance of 'polypeptide' near tyrosine spot and the inverse relationship of the intensity of the glutamic acid and 'polypeptide' spots. Also the progressive removal of all ninhydrin-positive material from the chromatogram following prolonged incubation at acid reaction. CT=control medium; 1-9, filtrates.
- Fig. 3. Chromatogram of the supernatants from washed, and also from washed and disintegrated cultures of *Staph. aureus* and *Esch. coli* showing the storage of amino-acids in the cell, the absence of polypeptides and the presence of glutamic acid in the disintegrate of *Staph. aureus* but not of *Esch. coli*. 1, washed *Staph. aureus*; 2, disintegrated *Staph. aureus*; 3, washed *Esch. coli*; 4, disintegrated *Esch. coli*.

(Received 26 November 1948)



MARJORY STEPHENSON (1885-1948)

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## Obituary Notice

### MARJORY STEPHENSON, 1885-1948

The death of Marjory Stephenson on 12 December 1948 has robbed biochemistry of a vigorous and productive exponent, and microbiology of a valuable interpreter of the chemical way of thinking. She was born on 24 January 1885 at Burwell, twelve miles from Cambridge; her life centred on Cambridge; she knew the town well and was well known in it. After graduating from Newnham she had hoped to complete a medical course but found it necessary to study Domestic Science at the Gloucestershire Training College instead, and she taught for a time there and at King's College of Household Science, London. Her career as a biochemist started at University College, London, working with R. H. A. Plimmer. In later life she always spoke of him with gratitude for providing her with this opportunity. She there studied the lactase of intestinal mucosa and showed that this enzyme was inhibited by glucose but not by galactose (Stephenson, 1911). She turned next to the synthesis of esters of palmitic acid (Stephenson, 1913) and then worked on metabolism in experimental diabetes (Moorhouse, Paterson & Stephenson, 1915). This work was interrupted by the 1914-18 war, during which she served with the Red Cross in France and at Salonika. After the war she returned to Cambridge and worked in the department of Frederick Gowland Hopkins on the fat-soluble vitamins (Stephenson & Clark, 1920; Stephenson, 1920). Hopkins in his wisdom encouraged her to leave the fields of animal metabolism and vitamins and to initiate a comprehensive study of the biochemical activities of bacteria.

There are obvious differences in the characters of recruits to different sciences at different stages of development and the Cambridge Biochemical Laboratory, which was for many years the main centre of biochemical teaching in Britain, attracted in its early days people who were vigorous, self-confident and not always tactful. In this environment M. S. (to use the title by which she became known internationally) was very much at home. Hopkins had established a new department and a new attitude of mind in Cambridge and his personal gentleness served admirably to weld the members of the department into a co-operative group. This was no mean feat, for they were once described to Hopkins by an important official of the University as 'That wrecking crew of yours'. Hopkins's character moulded the scientific outlook of his department but he came to depend, to an extent that was not always fully recognized, on M. S. for advice and support with the social and strategic problems of the department. Biochemistry met with strong and skilful opposition from many of the old-established departments—an opposition that still confronts those establishing biochemical schools in such Universities as lack them—and this antagonism was reciprocated by the research workers in the young department.



Some of the criticism had a superficial justice; the chemists said we lacked chemical skill, the biologists, biological knowledge. M. S. received, and returned with interest, criticism for her lack of fundamental bacteriological training. To her, in the first stages of her work, bacteria were simply tools and their taxonomy was of little importance. She recognized early that there could be greater metabolic differences between samples of the same culture at different phases of growth than between different species. The self-confidence that Pasteur had shown when, without medical training, he set about the construction of a bacteriological theory of disease, was an inspiration to her and she liked to quote his reply to someone who corrected him on a formal bacteriological point: 'If you only knew how little difference that makes to me.' As in Pasteur's case a new idea was of more importance than old knowledge and it is unlikely that her work would have been any more fruitful if she had had the training that many people looked on as essential.

Marjory Stephenson's new line of work at Cambridge was begun at a most exciting time: the Wieland-Thunberg theory of biological oxidations had been propounded; Hopkins had isolated what was thought to be an important hydrogen carrier, glutathione; and the Cambridge laboratory was turning to the study of intra-cellular enzymes with particular emphasis on oxidation mechanisms. This background had a profound and lasting effect on M. S.'s approach to bacterial metabolism.

Her first paper on bacteria, published in collaboration with Miss Whetham (Stephenson & Whetham, 1922), was concerned with fat formation by *Mycobact. phlei* and the effect of different media thereon. It was shown that acetate increased fat production more than any other substance tested. Methods were developed for the determination of both the respiratory quotient and the carbon balance-sheet during growth and it was found that, as the medium became exhausted, so the respiratory quotient fell and the stored fat disappeared (Stephenson & Whetham, 1923). These techniques were then applied to the study of the effect of oxygen on the metabolism of *Bact. coli communis* (Stephenson & Whetham, 1924).

During this period her colleagues J. H. Quastel and Margaret D. Whetham were developing the resting-cell technique by which, in conjunction with the Thunberg methylene blue procedure, they had demonstrated the presence of dehydrogenases in bacteria and had shown that the succinic dehydrogenase was reversible. This made possible an investigation of anaerobic growth. It was known that *Bact. coli* could grow aerobically but not anaerobically with succinate, lactate, glycerol or acetate as the carbon source, and also that certain organisms, including *Bact. coli*, could reduce nitrate to nitrite. In collaboration with Quastel and Whetham, M. S. showed that washed suspensions of *Bact. coli* oxidized leuco-methylene blue in the absence of oxygen, provided that either nitrate or chlorate were present. The coupling of succinate oxidation with nitrate reduction was then demonstrated and also that this coupled oxidation-reduction reaction would permit the anaerobic growth of *Bact. coli*: succinate was replaceable by lactate, glycerol or acetate; and fumarate, malate or aspartate could be substituted for nitrate as hydrogen acceptor. Chlorate,

whilst active as a hydrogen acceptor in cell-suspensions, could not be used as such in growth experiments owing to the toxicity of the chlorite formed as reduction product (Quastel, Stephenson & Whetham, 1925). These simple experiments served to emphasize the usefulness of washed suspension studies in the elucidation of problems of bacterial metabolism and at the same time made clear some of the principles underlying anaerobic growth. This line of work was continued with J. H. Quastel (Quastel & Stephenson, 1925) and later the effect of oxygen on the growth of obligate anaerobes was studied (Quastel & Stephenson, 1926).

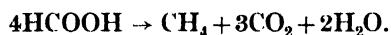
Many years later, at the inaugural meeting of the Society for General Microbiology in February 1945, M. S. analysed the steps in the development of research in the field of bacterial metabolism, and pointed out that research took place at a series of levels. At the first level the worker was concerned with mixed cultures; at the second with pure cultures growing in complex media; at the third with pure cultures growing in chemically defined media; at the fourth with washed cell-suspensions from pure cultures; and finally at the fifth level with cell-free enzyme preparations. No one level was, by itself, adequate; and for an understanding of bacteria as they are found in Nature, research must occur at all levels. Till about 1927 she had worked at levels two and four, but in 1928 she developed a method for obtaining intra-cellular enzymes (Stephenson, 1928). Thick suspensions of *Bact. coli* were allowed to autolyse in phosphate buffer at pH 7.4, and lactic dehydrogenase was found in the cell-free autolysate. Unfortunately the technique had only a limited application and further progress had to wait until improved methods for breaking-up bacterial cells were developed, some ten years later. In the meantime, work was continued with washed suspensions, and in collaboration with R. P. Cook (Cook & Stephenson, 1928) she made a detailed study of the oxidation of various compounds by *Bact. coli* and *Bact. alkaligenes*. This yielded the surprising result that, whereas formate was oxidized quantitatively to carbon dioxide and water, glucose, lactate, pyruvate or acetate were, as judged by the oxygen consumed, only partially oxidized; at the same time the substrate disappeared completely. This phenomenon could not be related to the viability of the suspension. Subsequent work in other laboratories and with other organisms confirmed these observations and demonstrated that only part of the substrate was oxidized completely, the remainder being assimilated by the cell.

About 1930 the Cambridgeshire Ouse was polluted by waste from a sugar-beet factory to such an extent that an active fermentation could be observed in the river itself. This provided an opportunity for investigating the methane fermentation, using the polluted river water as an inoculum. These enrichment cultures, in addition to producing methane from formate, reduced sulphate to hydrogen sulphide and made methane from carbon dioxide and hydrogen. Stephenson & Stickland (1931*a*) commented on these observations as follows: 'This led to the conception that carbon dioxide and sulphate were acting as hydrogen acceptors in a system where molecular hydrogen was the hydrogen donor and it seemed likely that bacteria were present in the mixed culture capable of activating hydrogen.' To test this hypothesis a washed suspension of

the enrichment culture was examined by the Thunberg method for its ability to activate hydrogen. It was found that, in the presence of hydrogen, a rapid reduction of methylene blue occurred. A coliform bacterium was isolated from the crude culture and was shown to contain the enzyme, which was named hydrogenase. This enzyme was found to be widely distributed amongst bacteria, and in view of this it was suggested, somewhat tentatively, that the production of hydrogen from formate, a reaction which L. H. Stickland (1929) had been investigating, involved two enzymes, formic dehydrogenase and hydrogenase.

The reduction of sulphate to hydrogen sulphide by hydrogen was next investigated (Stephenson & Stickland, 1931*b*) and a bacterium closely related to *Desulphovibrio desulphuricans* was isolated in pure culture. The bacterium possessed a powerful hydrogenase and could grow on sulphate and hydrogen with carbon dioxide as carbon source; sulphite or thiosulphate could replace sulphate as hydrogen acceptor. This work provided the first indication that this group of organisms could live autotrophically.

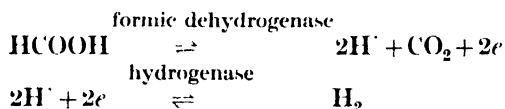
Finally the methane organisms were examined (Stephenson & Stickland, 1933*a*). Enrichment cultures were readily maintained on formate and produced methane according to the following equation:



A pure culture was isolated by the single-cell method, since conventional plating techniques consistently failed, and the organisms so obtained were found to possess hydrogenase. A number of one-carbon compounds, including formate, formaldehyde (added as hexamethylenetetramine), methanol, carbon monoxide and carbon dioxide, were reduced to methane in the presence of hydrogen by this organism; in addition sulphate was reduced to hydrogen sulphide. Though possessing some of the characteristics of the methane bacteria subsequently isolated by Barker and by Schnellen, this organism was unique in its ability to reduce methanol, formaldehyde and formate to methane, and to-day it seems probable that in spite of all efforts to purify it the culture was contaminated with a sulphate-reducer (the reduction of sulphate to hydrogen sulphide supports this view). The sulphate-reducer would produce carbon dioxide from the above substrates, which carbon dioxide would then be reduced to methane by the methane bacteria.

At this point the work along these lines was dropped and a re-investigation of the production of hydrogen from formate was begun (Stephenson & Stickland, 1932). L. H. Stickland (1929) had shown that washed suspensions of *Bact. coli* grown on a tryptic digest of casein medium, possessed a powerful formic dehydrogenase, but would produce hydrogen from formate only after a prolonged incubation with the substrate; it seemed as though hydrogen production were associated with the growth of the organism. The appearance of Karström's paper (1930) on adaptive enzymes prompted the suggestion that the production of hydrogen and carbon dioxide from formate might be an adaptive phenomenon; accordingly *Bact. coli* was grown on media containing formate and it was found that washed suspensions of these 'adapted' cells decomposed formate to hydrogen and carbon dioxide. Enzymes producing

hydrogen were called hydrogenlyases, and the formic enzyme, formic hydrogenlyase, to distinguish it from glucose hydrogenlyase, an enzyme considered to produce hydrogen specifically from glucose; two distinct enzymes were postulated on the grounds that the substrate affinity curve and the pH curve for the two processes differed from one another. This view met with considerable opposition and it now appears unlikely that there are, in fact, two hydrogenlyases in *Bact. coli*. In an earlier paper Stephenson & Stickland had suggested that the production of hydrogen from formate might be explained in terms of a coupled reaction between formic dehydrogenase and hydrogenase:



If this were true, it was clear that all organisms producing hydrogen from formate must contain both hydrogenase and formic dehydrogenase. This was not the case; four strains of *Bact. lactis aerogenes* were found which made hydrogen from formate yet contained no hydrogenase, and it was concluded that formic hydrogenlyase was in fact a separate enzyme. The adaptive nature of formic hydrogenlyase was established by Stephenson & Stickland (1933*b*), for the enzyme was produced only when the medium contained formate and under conditions of partial anaerobiosis; further, enzyme production seemed to be independent of growth.

This work on adaptive enzymes turned M. S. to the study of the factors involved in enzyme formation, but hydrogen metabolism was not dropped. D. D. Woods (1936) showed that formic hydrogenlyase was reversible and Woods & Clifton (1937) studied hydrogen formation from amino-acids and demonstrated that formate was not an intermediary. In 1937 M. S. reviewed the position of formic hydrogenlyase (Stephenson, 1937) and passed her final judgment on hydrogen metabolism in her contribution to the volume dedicated to A. J. Kluyver (Stephenson, 1948).

The formation of galactozymase in the presence of the substrate by *Saccharomyces cerevisiae* was investigated by Stephenson & Yudkin (1936) and in *Bact. coli* by Stephenson & Gale (1937*a*).

M. S. had always been interested in the amino-acid metabolism of bacteria, and under her guidance L. H. Stickland, and later D. D. Woods, elucidated the metabolism of *Cl. sporogenes*. It had been widely held that glucose exercised a 'sparing' action on amino-acids; this she disbelieved, and with E. F. Gale undertook the study of the enzymes oxidizing amino-acids (Stephenson & Gale, 1937*b*). The method of attack was to examine the effect of different media and conditions of growth on the formation of the alanine, glycine and glutamic deaminases. In all cases the presence of glucose in the medium inhibited the formation of the enzyme. This inhibition was due neither to the anaerobic conditions set up by the resulting fermentation nor to the increase in hydrogen ion concentration. The enzymes, once formed, were not markedly affected by glucose.

The serine deaminase was next investigated (Gale & Stephenson, 1938). The

enzyme resembled those previously studied in that its formation was inhibited by glucose, but it differed in that the deamination occurred both aerobically and anaerobically, and was much more rapid than those previously studied. The enzyme was not stable; a suspension allowed to stand even at 0° lost its activity. This inactivation could be prevented by the addition of extracts of *Bact. coli*, or by such reducing systems as hydrogen, formate, glutathione, all of which required phosphate for maximum activity. Reactivation after decay had taken place could only be brought about if the decay took place at 0°, and systems similar to those preventing decay also restored activity.

In collaboration with A. R. Trim (Stephenson & Trim, 1938), the deamination of adenylic acid, adenosine and adenine was investigated. Adenylic acid was both deaminated and dephosphorylated; adenosine was rapidly deaminated whereas adenine was attacked but slowly, this last reaction being stimulated by adenosine.

Meanwhile V. H. Booth and D. E. Green had made their wet-crushing mill which made possible the preparation of cell-free enzymes from bacteria. With this new tool M. S., with E. F. Gale and J. L. Still, made cell-free preparations of a number of enzymes from *Bact. coli* (cf. Gale & Stephenson, 1938; Stephenson, Gale & Still, 1939).

When the second world war started, the possibility of a rubber shortage and the probable extension of the synthetic rubber industry stimulated M. S. to study the mechanism of the butanol fermentation, with a view to increasing the yield of solvents. This fermentation had never been examined by the use of washed cell-suspensions. Great difficulty was experienced in the preparation of active suspensions, for the enzymes involved were very labile. The inclusion of both glucose and yeast autolysate in the suspending medium, and careful attention to anaerobiosis throughout the preparation of suspensions finally gave active preparations. This work was done with R. Davies as her co-worker (Davies & Stephenson, 1941).

The microbiological assay of vitamins of the B group next engaged her attention, and although M. S. published nothing on this, she took an active part in the large-scale trials of the various methods, thought necessary as a result of the divergent values for the same materials reported by different laboratories. She did not enjoy this work, though the full-cream dried milk left over from the experiments was some compensation.

When the war ended, M. S. became interested in the synthesis of acetylcholine by micro-organisms. There were reports in the literature of the production of acetylcholine during the sauerkraut fermentation; this was confirmed (Stephenson & Rowatt, 1947), and the responsible organism isolated from sauerkraut. The presence of choline in the medium was obligatory for the synthesis and, following up F. Lipmann's observations on acetylation by the liver, it was demonstrated that pantothenic acid also was a component of the system.

Her last piece of work, not yet published, was concerned with nucleic acid metabolism; soon after she had begun this problem she wrote... 'I have got onto the most interesting piece of research I have ever done and where it's going to turn next I just don't know.'

Her research work may be summarized as the application to bacteria of Hopkins's concept of dynamic biochemistry; her tools were washed cell-suspensions and, where possible, cell-free extracts. She was not particularly interested in complex reactions, at least until her last years, and in general she attempted to study single enzymes. Her approach was simple: to demonstrate the reaction with washed cell-suspensions; to study the kinetics of the system and the factors controlling the formation of the enzyme; and finally, in order to learn more about the mechanism, to try to prepare an active cell-free extract. This was the method developed by her and now used wherever bacterial metabolism is studied.

She spread her gospel in a number of ways, not the least of which was her course given to the Part II Tripos class in biochemistry at Cambridge. It is a testament to her activities that bacterial metabolism flourished in Cambridge to such an extent that it was recognized by the University as a discipline in its own right, and she herself was made University Reader in Chemical Microbiology in 1947.

She approached bacteriology without, in the first instance, any regard for its practical applications and the support for her work by the Medical Research Council from 1922 and her establishment on its Staff in 1929 were instances of the breadth of view of that body. In the first edition of *Bacterial Metabolism* (1930) it is clear that the paucity of references to pathogenic bacteria is due rather to absence of information on these organisms than to avoidance of them by the authoress. She took the view that her business was to deal with bacteria 'as living organisms apart from their role as disease germs', but, as her second edition (1939) records, disease germs had in the meantime disclosed characters which made them eminently suitable for study as living organisms. Thus, as time went on her interests tended to embrace medical bacteriology to an ever-increasing extent, and though she herself did little work with pathogenic bacteria, she certainly had an influence in creating that wider outlook which is notable in present-day teaching in medical bacteriology.

In harmony with her views of the wide discipline of microbiology as a whole, M. S. took an active part in founding the Society for General Microbiology, and attending numerous preparatory committee meetings between November 1943 and February 1945, when the Society was formally inaugurated. She was an Original Member, served on the Committee of the Society from its foundation, and was unanimously elected as the Society's second President in September 1947, which office she held at the time of her death. She attended her last Committee meeting only a few weeks before she died.

The monograph, *Bacterial Metabolism* (1930, 1939, 1949), enabled M. S. to reach a wider public, and in this she was aided by a lucid and forceful style; it is a very personal book. The subject is presented in terms of enzyme mechanisms and she has little to say on those aspects which at the time of writing were not amenable to this treatment. It is interesting to compare the three editions of *Bacterial Metabolism* with this in mind, and to observe the succession of problems which have been answered in these terms. The book has been criticized on the grounds that it represents bacteria as 'little bags of enzymes'. In reply

it may be asked, how else can bacterial metabolism be described save in terms of the actions of enzymes? *Bacterial Metabolism* rendered a very great service by introducing to a wide public a new approach in the study of bacteria. In addition to this book she wrote the articles on Bacterial Chemistry in the first four volumes of the *Annual Review of Biochemistry*, thereby helping to keep biochemists in touch with what she considered to be the important advances in her own subject. The article of which she was proudest, and over the writing of which she took great pains, was her obituary of Hopkins for the *Biochemical Journal*; when she had finished it she exclaimed 'I feel I know Hoppy now', and he was, of course, her hero.

Marjory Stephenson's scientific outlook was strictly empirical. In her own field she kept her attention firmly on the actual observations and was less interested in the theories that flowed from them. This made her impatient with arguments that depended on the fitting of equations to observed curves; she used mathematics as a tool rather than as a guide. A quotation from the second edition of *Bacterial Metabolism* illustrates her attitude:

In the problem of bacterial growth advances have been made along new lines. Happily this subject now attracts mathematicians and statisticians less than formerly but has passed into the hands of biochemists interested in problems of nutrition; this has led to results of both theoretical and practical importance and has revealed *inter alia* that the complex and peculiar media employed by bacteriologists in the cultivation of 'difficult' pathogens are rendered necessary owing to the inability of many parasitic organisms to synthesise for themselves certain molecules essential for growth.

The point also figures in some of her, lamentably infrequent, contributions to *Brighter Biochemistry* (a laboratory journal published in Cambridge). Her arguments, written or verbal, depended on the assumption that a bacterium does not bring about actions by accident but is adapted for survival in some of the environments it may meet in Nature and that it is unlikely to retain a given capacity, against the flood of mutations and variations, unless it has survival value on occasion. M. S. was safeguarded from being led by physiology into simple teleology by this awareness of selection and of the probability that many enzymes do not bring about the same action in Nature that is studied in the laboratory. Enzyme specificity is never complete and in the economy of the bacterium an enzyme may be occupied with a different substrate or with catalysing an action in the reverse direction. In the environment supplied by Hopkins's dynamic approach to biochemistry her physiological interest led her naturally to the study of adaptive enzymes and she liked to emphasize that they were a feature of microbial rather than vertebrate economy, because the former was suited to a variable and varying environment, whereas the latter maintains a relatively constant internal environment for its enzymes.

Marjory Stephenson adopted much the same attitude towards people as she adopted towards science. She was concerned with what they were actually doing and with their motives rather than with what they said they were doing and why. This pursuit of personal information and discussion of motive, especially when undertaken by someone with her infectious gaiety, could become formally indistinguishable from gossip and the pejorative word was

sometimes used by those whose activities were being analysed. The analysis of motive was however a necessary means towards the end of planning an environment in which research workers could flourish. She knew that it was only when one had begun to understand the motives that had led a person into research that one could give any useful advice if he seemed not to be making a success of it. She was unsparing in her condemnation of secretiveness, personal vanity and competitiveness in scientists and for this reason jeered at most of the medals and awards that scientists on occasion confer on one another. Although she realized that it was probably unavoidable, she looked on many of the consequences springing from the existence of bodies of limited membership, such as the Royal Society, as unfortunate. Each year when a new list of Fellows was published she would remark '...that means a few more scientists can settle down to their work instead of fussing about their reputations'. In this context another comment of hers should be preserved: 'These young men fuss about their reputations as if they were ageing virgins in a Victorian novel.' The various disabilities to which women scientists are subjected were, in her opinion, almost compensated for by their freedom, between the ages of 35 and 50, from this anxiety. As a feminist she was pleased when the old anomalous rule that women could not be Fellows of the Royal Society was abolished and she was human enough to be gratified that she was one of the first to be admitted. Her pleasure was however marred by the realization that she might be accused of inconsistency on what had been almost a matter of principle.

Outside the laboratory M. S. had many interests. She was widely travelled and widely read and it would have been difficult to find a conversational theme that would not have interested her. She was a fellow of Newnham College and spent a considerable amount of time working and thinking for the College and acting on those committees to which she could contribute. Politics interested her greatly. As might be expected in a person of her independence of mind, she did not adhere docilely to any party but supported particular activities of whatever party was being most useful at the time. Her main political activity was in the period 1931–1937 when she gave valuable help with advice, money and hospitality to those anti-war movements that looked on war as a manifestation of economic and political imbalance. She was satisfied that if politics were the cause of war the cure must be political too. At the time of her death she was a vice-president of the Association of Scientific Workers and had on many occasions been a source of strength to it; she was, for example, a guarantor during the period when its finances were rather insecure.

Gardening always interested her; at the house in which most of her life in Cambridge was spent the opportunity was poor, but when she moved to more open surroundings her garden gave her intense satisfaction. She believed strongly in its psychotherapeutic value and attributed the relatively low crime and suicide rates in Britain to our national preoccupation with gardening. She often suggested that if those scientists whose behaviour did not come up to her standards would only undertake the care of a garden they would be much improved. From time to time she painted, but apparently with less



satisfaction, for she was always unwilling to show her pictures to those who might be critical.

It is difficult to imagine what a protracted old age might have been like; she had so many interests—and would probably have developed more—that it would not have been notably peaceful. 'Middle aged' was a term that she used contemptuously of some of her contemporaries and juniors. She herself escaped it and passed quickly from youth to old age after her first operation for cancer. Soon after this operation she knew that death could not be long postponed but had the wisdom and courage to remain gay, argumentative and active to within a few weeks of her death.

S. R. Elsdon

N. W. Pirie

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## Flagellation of Certain Species of *Pseudomonas* as seen with the Electron Microscope

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**SUMMARY:** The number and distribution of flagella on six species of *Pseudomonas* varied considerably for different cells of a culture. The flagellation reported for certain species of *Pseudomonas* in the sixth edition of Bergey is in error. A flagellum on a *Pseudomonas* cell might originate laterally or terminally. In some electron micrographs the flagellum was apparently inserted into the cell and attached to the cytoplasm. The diameter of the flagella varied greatly, and there was a tendency for certain of them to stick together in bundles. Some pictures showed evidence for the tubular nature of flagella, but this may have been an artefact. The terms monotrichate, lophotrichate, and amphitrichate are of little use as an aid in identifying a bacterial species, and bacteria should be classified as motile or non-motile, and if motile as possessing either peritrichate flagellation or with a tendency for terminal flagellation.

The electron microscope has been very useful to bacteriologists as a tool for the study of bacterial morphology, particularly in the study of bacterial flagellation. The techniques are simpler than the usual flagella staining methods, and the results are certainly as reliable. Often many more flagella are observed with the electron microscope than were previously reported by workers using the flagella staining techniques (see Hofer, 1944).

Two widely different methods are often used in electron microscope studies. One involves the use of the shadowing technique as outlined by Williams & Wyckoff (1945). The other simply records electron-transmission and electron-scattering of the object itself. The shadow technique has the advantage of giving the picture a third dimension and aids greatly in showing the actual three-dimensional shape of an object, or to detect superimposed objects such as flagella passing over the surface of the bacterial cell. The shadow technique has the disadvantage that internal structure of the cell is hidden except as it might appear as a bump or protrusion on the surface of the cell. The simpler technique of recording electron-transmission and scattering by the object has the advantage of actually recording the internal structure of the dried cell. There has recently been a great tendency to ignore the latter technique in the study of bacterial morphology. The present paper deals with the number and origin of flagella of certain *Pseudomonas* species. The shadow technique was not used, principally because the necessary equipment was not available at the time the work was done.

Previous studies on the flagellation of the genus *Pseudomonas* have used staining techniques. The reports have been confusing. Reid, Naghski, Farrell & Haley (1942) reported that all of 27 strains of *Pseudomonas* which showed monotrichate flagellation belonged to the species *aeruginosa*, while 608 strains showing two or more flagella all belonged to the species *fluorescens*. Harris

(1940) reported that of 80 monotrichate strains of *Pseudomonas* studied, most were *Ps. aeruginosa* and a few were *Ps. fluorescens*. The sixth edition of Bergey (1948) reports *Ps. fluorescens* as being motile, possessing a polar flagellum, and *Ps. aeruginosa* as possessing one to three polar flagella in one sentence, and as being monotrichate in the next sentence. This same confusion exists for many other motile species of bacteria. Extensive electron microscope studies are obviously necessary to clarify and extend our knowledge of bacterial flagellation.

#### EXPERIMENTAL METHODS

Table 1 lists the twelve strains of *Pseudomonas* spp. studied. The cultures were grown on nutrient agar slopes at 37° for 48 hr. and suspended in distilled water. The suspension was diluted to give well-isolated cells on the slide. This was necessary because more than one cell in a low-power field made it difficult to determine the origin of a particular flagellum. During the preparation of the suspension care was taken not to agitate the cells unnecessarily, and thus to risk breaking flagella from the cells. Collodion slides were prepared by placing a drop of the dilute suspension on the collodion-covered screen, and after a few seconds shaking off the excess. The slide was then allowed to dry in air. A 50kV. RCA, EMU Model, electron microscope was used. Observations were made until variation in the number of flagella was demonstrated for each strain, or until it was felt that variation could not be demonstrated under the conditions of study.

#### RESULTS AND DISCUSSION

Table 1 presents the results obtained with the twelve strains of *Pseudomonas*. Six species were represented and in all except *Ps. denitrificans*, the flagella varied from one to several per cell. This does not prove that *Ps. denitrificans* is always monotrichate, because other strains and conditions of cultivation were not studied. One strain of *Ps. fluorescens* seemed to be monotrichate, while other strains were lophotrichate. Comparison of these results with the type of flagellation recorded in Bergey (1948) shows that wherever Bergey's manual has made a specific statement, such as 'possessing a single polar flagellum', this study has shown the statement to be incorrect.

The members of the genus *Pseudomonas* are generally thought of as having terminal flagella. In this study flagella frequently appeared to originate on the sides of the cell, or even on opposite ends of the cell. Figs. 9, 10, 12, and 13 in Plates 2 and 3 show the origin of flagella over a wide area of the end of the cell. Two definitely lateral flagella can be seen in Pl. 3, fig. 13. Pl. 3, figs. 14, 15, show a single flagellum originating on the lateral part of the cell. One cell in Pl. 3, fig. 15, has a rather vague object coming from it which is probably not a flagellum. Pl. 3, fig. 16, shows two flagella originating from the lateral surface of the cell which join to form one large single flagellum. Pl. 2, figs. 7, 8, show an amphitrichate arrangement of the flagella. In Pl. 2, fig. 8, the flagella are thick, in that they measure 0.026  $\mu$  in diameter, while those in Pl. 2, fig. 7, are very fine and measure only 0.016  $\mu$  in diameter. Note also in Pl. 2, fig. 7, that two flagella appear to join to form one flagellum. In all of

these pictures well-isolated cells are shown, and there is little chance that the flagella seen might originate from neighbouring cells. It is possible, however, that the lateral flagella in Pl. 3, figs. 14, 15, might have originated on the end of the cell and fold under the cell to emerge on the side. The apparent

Table 1. *Number of flagella observed on Pseudomonas*

| Organism   | Flagellation as reported in<br>Bergey (6th ed.) | Flagellation as observed<br>with electron microscope<br>(present work) |               |
|--|---|--|---------------|
|  |   | No. of cells   | Flagella/cell |
| <i>Ps. ovalis</i><br>A.T.C. No. 8209   | Motile, possessing a single<br>polar flagellum  | 5  | 1             |
|  |   | 4  | 2             |
|  |   | 1  | 4             |
|  |   | 1  | 5             |
|  |   | 1  | 6             |
| <i>Ps. mildenbergii</i><br>A.T.C. No. 795  | Motile, possessing polar fla-<br>gella          | 6  | 1             |
|  |   | 3  | 4             |
|  |   | 1  | 6             |
|  |   | 1  | 7             |
|  |   | 1  | 15            |
| <i>Ps. denitrificans</i><br>A.T.C. No. 8453  | Motile  | 20   | 1             |
| <i>Ps. putida</i><br>A.T.C. No. 4359   | Motile, possessing polar fla-<br>gella          | 11   | 1             |
|  |   | 5  | 2             |
|  |   | 1  | 3             |
|  |   | 1  | 5             |
|  |   | 1  | 6             |
| <i>Ps. fluorescens</i><br>A.T.C. No. 9721<br>Stone R 1<br>U.S.C.                       | Motile, possessing a polar<br>flagellum         | 15   | 1             |
|  |   | 20   | 1             |
|  |   | 4  | 2             |
|  |   | 14   | 1             |
|  |   | 2  | 2             |
|  |   | 1  | 6             |
|  |   |  |               |
| <i>Ps. aeruginosa</i><br>Stone 96b<br>Stone 41a<br>U.S.C. 46<br>U.S.C. A1<br>U.S.C. B1 | 1 to 3 polar flagella                           | 30   | 1             |
|  |   | 5*   | 2             |
|  | Monotrichous (Reid, <i>et al.</i> )             |  |               |
|  |   | 11   | 1             |
|  |   | 6  | 1             |
|  |   | 5  | 1             |
|  |   | 1  | 3             |
|  |   | 7  | 1             |
|  |   | 2  | 2             |
|  |   | 1  | 3             |

\* Also very numerous 'pseudo' flagella.

A.T.C. = American Type Culture.

Stone = R. W. Stone, Penn. State Univ.

U.S.C. = University of Southern California.

lateral origin of a flagellum was frequent, and of 220 flagellated cells twenty-one had lateral flagella. This indicates that the origin of flagella for various species of the genus *Pseudomonas* might not be restricted to the terminal area of the cell or only to one end of the cell.

Pl. 2, fig. 6, shows many small flagella-like structures originating from the end of the cell opposite the end with a large flagellum. These structures were very common on the cells from *Ps. aeruginosa* strain 96b. On observing this culture in a hanging drop preparation, it was found that this strain possessed a remarkably high degree of motility. Pls. 1, 2, figs. 4, 5, show more of these fine flagella-like structures. Their origin appeared to be at any point on the cell.

The demonstration for the genus *Pseudomonas* of the lateral origin of flagella, the amphitrichate flagellation, and the fine flagella-like structures occurring almost anywhere on the cell, is compatible with the 'flagella degeneration' theory of Conn *et al.* (1920, 1938, 1940, 1947). Conn has stated that perhaps all motile bacterial cells were at one time peritrichate; hence, a monotrichate or lophotrichate cell has merely lost the ability to produce flagella in certain areas. Thus, any area of the cell might possess the ability to produce a flagellum. This theory might also explain the fine flagella-like structures seen in *Ps. aeruginosa* 96b, in that they might represent 'degenerate' flagella; but this is pure speculation. The present paper does not attempt to prove these structures to be flagella. There is some possibility that they might represent 'Pseudo-flagella', as described by Thjotta & Kåss (1946), in which case they might be simply strung-out capsular material. Supporting evidence that they might be flagella consisted of the extremely high motility of the cells of this culture, as compared with the cells of other *Pseudomonas* cultures which did not show these structures.

The variation in numbers and distribution of flagella, as reported above, throws doubt on the usefulness of such terms as monotrichate, lophotrichate and amphitrichate, when these terms are used to aid in the classification. It would seem best simply to refer to bacteria as being motile or non-motile; and if motile, as possessing either peritrichate flagellation or a tendency toward terminal flagellation.

Pl. 1, figs. 1, 2, 3, show an apparent origin of the flagellum from the inner cytoplasm of the cell. The flagellum can be seen apparently to penetrate through the cell-wall, and then to expand into a broad base as it joins with the cytoplasm. This observation was made on only a few of the cells. The fact that this insertion could not be seen for all flagella is explicable on the basis of the drying effect of the electron microscope which causes the cytoplasm to retract from the cell-wall and the flagella to break away from it. Sometimes, by luck, the insertion remained intact. Recently van Iterson (1947) has published electron micrographs using the shadow-cast technique, which appear clearly to show a similar internal origin of flagella for *Vibrio metchnikovii* and *Spirillum serpens*. However, in both the pictures presented in the present paper, and in the pictures presented by van Iterson, there is the possibility that the flagella are merely underlying the cell-wall and that they really originate from the outer part of the cell; thus the apparent internal origin would be an artefact. Johnson, Zworykin & Warren (1948) presented electron microscope evidence to show that bacterial flagella did penetrate through the cell-wall. They showed that cytoplasm of certain cells of *Achromobacter* spp. had flagella attached even

though the cell-wall had apparently been removed. They also found, however, fragments of cell-wall, devoid of cytoplasm, with attached flagella. They proposed a theory that flagella might be composed of elements from both the cell-wall and the cytoplasm. Leifson (1931) also presented some evidence, not obtained by the electron microscope, which seems to rule out the capsular origin of flagella in the manner proposed by Pijper (1947). Leifson showed that flagella appeared and began to grow in length while the spores of *Bacillus cereus*, *B. vulgaris* and *B. flavus* were in the early stage of germination and before the cell had acquired much, if any, motility. It could be that confusion is occurring simply because Pijper generalized observations based on a single species of bacteria.

Pl. 3, fig. 11, shows a flagellum which has split into two smaller flagella, Pl. 2, figs. 9, 10, show bundles of flagella which split into many smaller units. Practically every publication with electron microscope pictures of motile bacteria has shown the existence of 'split' flagella. Obviously, bacterial flagella tend to stick together when they are in juxtaposition. Pl. 2, figs. 7, 8, as commented upon earlier, obviously show the existence of flagella of different thicknesses. These flagella, as far as could be determined, were single flagella. The observation of variable thickness, and the sticking together of flagella, is in agreement with the observations of Johnson, Zworykin & Warren (1943) in their studies of the flagellation of cells of luminous bacteria.

Pls. 1, 2, figs. 4, 5, show a surprising indication of structure for a flagellum. The pictures suggest either a tubular structure, or two flagella held together by a fine sheath. The existence of tubular structure in bacterial flagella is a matter of considerable debate. Polevitsky (1941) reported the apparent tubular structure of flagella in her electron microscope study of several unnamed genera of bacteria. Mudd & Anderson (1942) made similar observations for *Eberthella typhosa* treated with 0.59 M lead acetate. However, they believed the appearances to be artefacts and the structure to be due to the accumulation of dissolved protoplasmic components along the sides of the flagella during the drying of the preparation. This contention was supported by the observation that the tubular structure occurred only in the areas adjacent to what were termed 'lysed' cells. The same flagellum would appear tubular near the cell, but structureless at a distance from the cell. It is possible, however, to interpret their pictures differently; in the area adjacent to the cell the lead acetate might be in higher concentrations, and this, as shown in an earlier section of their paper, might increase contrast so as to make the tubular structure appear. The cells pictured in the present paper were in no way treated; nevertheless the tubular structure appears throughout the length of the flagellum, and the background is clear. Moreover, if the dark edges were due to accumulated dirt, then this line might also be expected to occur along the edge of the cell-wall; but this was not so. It is also possible that the double structure could be two small flagella lying parallel and held together by a thin sheath. The joining of two flagella in Pl. 3, fig. 16, lends support to this explanation, but it seems improbable, since many 'tubular' flagella were seen with no indication of the joining of two flagella at the base. The exact interpretation of

this appearance, then, is in doubt, and the necessity for more work along these lines is strongly indicated.

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## EXPLANATION OF PLATES

### PLATE I

- Fig. 1. *Ps. aeruginosa* (96b). Showing apparent origin of a flagellum from inside of cell.  $\times 82,000$ .
- Fig. 2. *Ps. aeruginosa* (96b). Showing apparent origin of a flagellum from inside of cell.  $\times 130,000$ .
- Fig. 3. *Ps. denitrificans* (A.T.C. No. 8453). Showing apparent origin of a flagellum from inside of cell.  $\times 95,000$ .
- Fig. 4. *Ps. aeruginosa* (96b). Showing apparent tube-like structure of large flagella, also very fine flagella-like strands.  $\times 120,000$ .



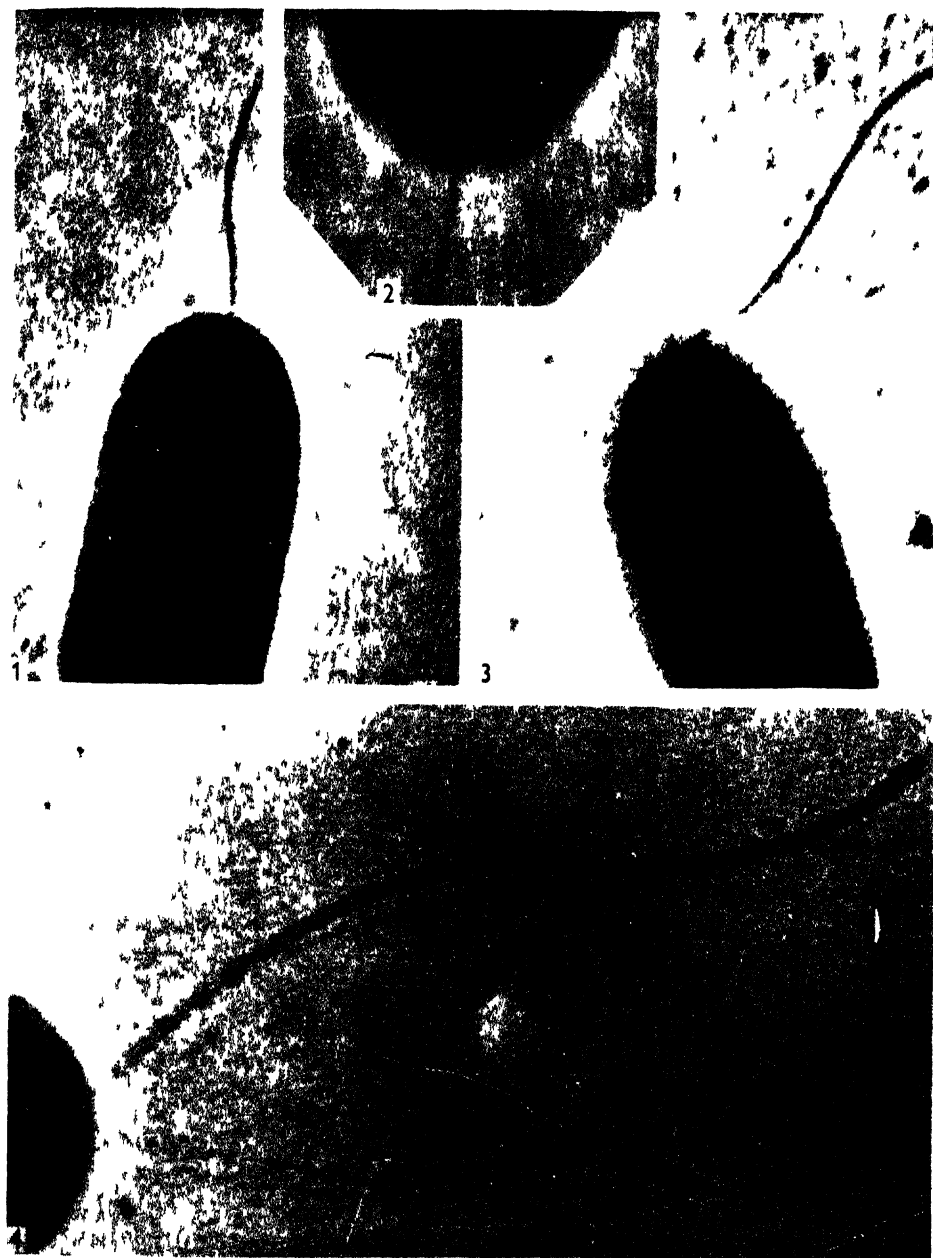
## PLATE 2

- Fig. 5. *Ps. aeruginosa* (96b). Showing apparent tube-like structure of large flagella, also very fine flagella-like strands.  $\times 52,000$ .
- Fig. 6. *Ps. aeruginosa* (96b). Showing many fine flagella-like structures on one end of cell.  $\times 30,000$ .
- Fig. 7. *Ps. aeruginosa* (U.S.C. A1). Showing amphitrichate arrangement of flagella.  $\times 38,000$ .
- Fig. 8. *Ps. putida* (A.T.C. No. 4359). Showing amphitrichate arrangement of flagella.  $\times 38,000$ .
- Fig. 9. *Ps. mildenbergii* (A.T.C. No. 795). Showing a bundle of flagella splitting into separate units.  $\times 42,000$ .
- Fig. 10. *Ps. mildenbergii* (A.T.C. No. 795). Showing the splitting of bundles of flagella into separate units.  $\times 52,000$ .

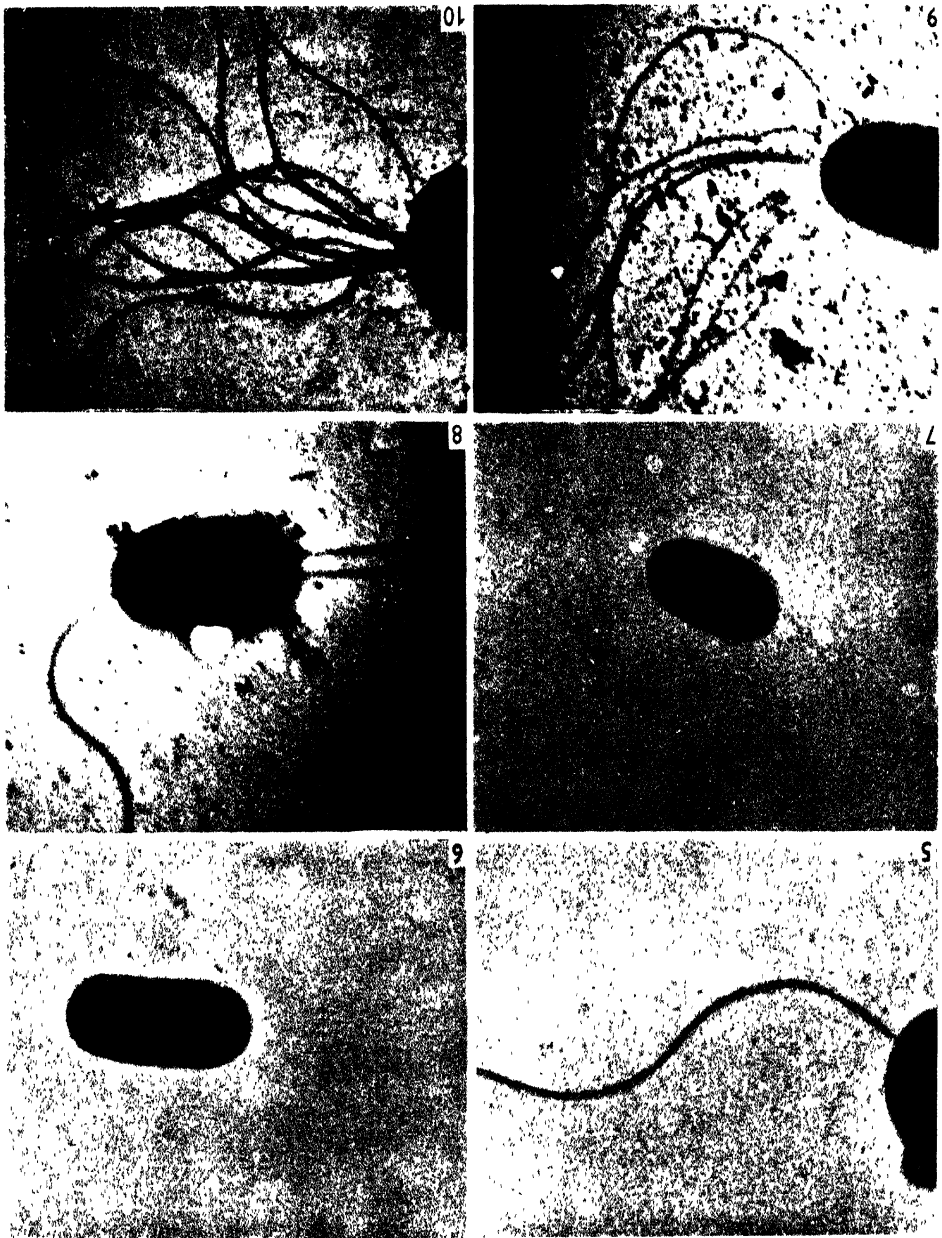
## PLATE 3

- Fig. 11. *Ps. aeruginosa* (U.S.C. A 1). Showing split flagellum, also cell vacuole.  $\times 28,000$ .
- Fig. 12. *Ps. putida* (A.T.C. No. 4359). Showing the origin of flagella over a wide area on the end of a cell.  $\times 38,000$ .
- Fig. 13. *Ps. mildenbergii* (A.T.C. No. 795). Showing lateral origin of flagella.  $\times 32,000$ .
- Fig. 14. *Ps. aeruginosa* (96b). Showing lateral origin of a flagellum.  $\times 34,000$ .
- Fig. 15. *Ps. aeruginosa* (96b). Showing lateral origin of a flagellum.  $\times 38,000$ .
- Fig. 16. *Ps. mildenbergii* (A.T.C. No. 795). Showing lateral flagella uniting to form one flagellum.  $\times 34,000$ .

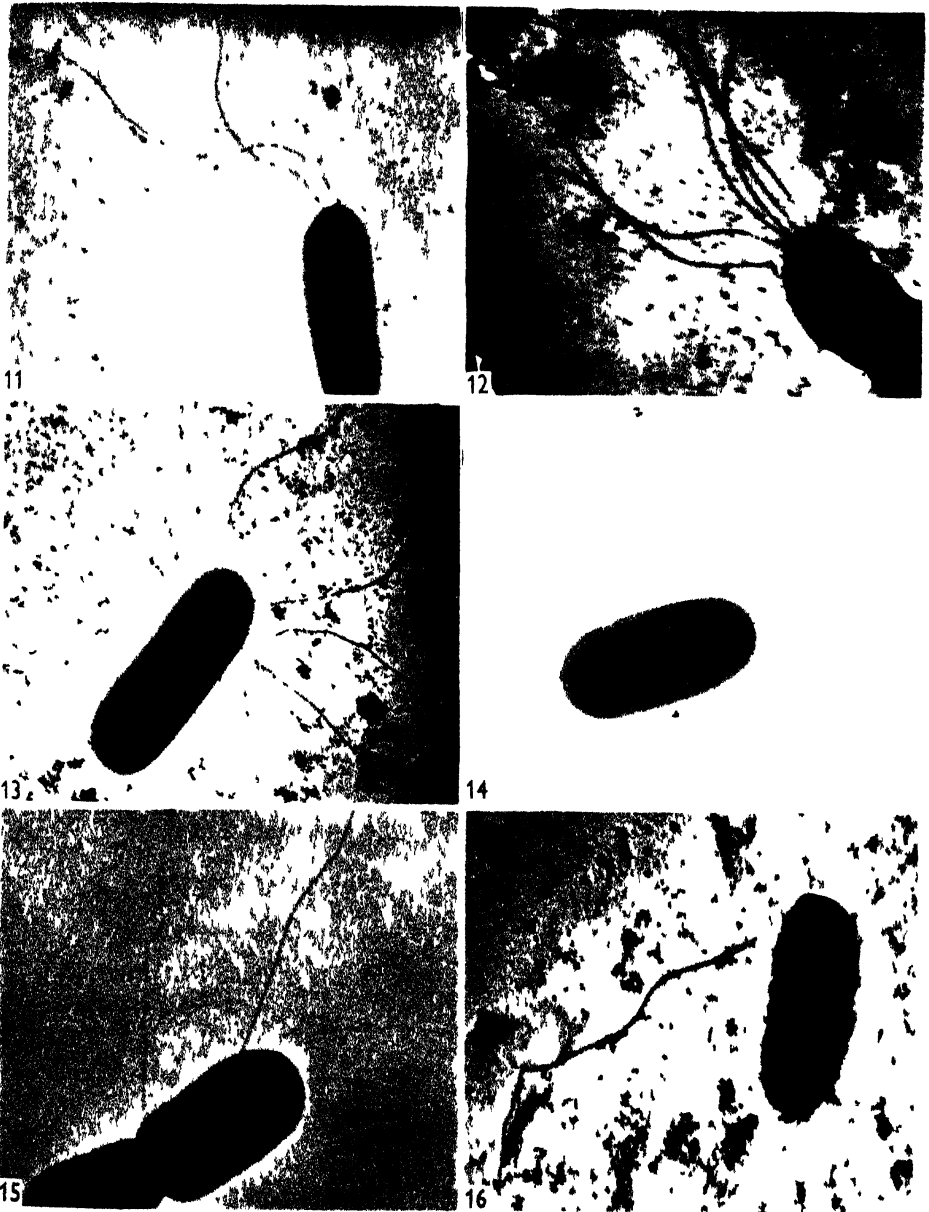
(Received 9 October 1948)



Figs 1-4



Figs 5-10



Figs. 11-16



## The Biological Assay of Penicillin by a Modified Plate Method

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**SUMMARY:** The general form of assay for streptomycin described by Brownlee, Delves, Dorman, Green, Grenfell, Johnson & Smith (1948), based on an  $8 \times 8$  quasi-Latin square layout of cavities on a large agar-covered glass plate, in place of Petri dishes, can be adapted to penicillin. A new design, using the principle of double confounding, allows the estimation by the usual four-point assay, of seven (instead of three) unknown solutions per plate of 64 holes. In routine use this assay gives fiducial limits ( $P=0.95$ ) of about  $\pm 9.6\%$ , including dilution errors.

The assay procedure previously described in this Journal (Brownlee *et al.* 1948) for streptomycin, is immediately adaptable to penicillin. For dealing with large numbers of fermenter samples in particular, we have found modifications desirable, which we describe in the present paper.

### *Experimental design*

The basic plate takes 64 holes arranged in an  $8 \times 8$  square. For the streptomycin assay, Yates's (1937) arrangement of a quasi-Latin square was used to assay three unknown solutions in terms of a standard, all solutions going on the plate at two dilutions in a certain order designed to eliminate bias arising from the time taken to complete the filling of the plate. A slightly less accurate assay was desired which would allow us to test considerably more than three 'unknowns' per plate. The solution of the problem in statistical design is as follows.

Fisher (1942) gives an account of the theory of double confounding, in which two restraints can be imposed on the layout of the factorial experiment. For the 2<sup>8</sup> experiment, Brownlee (1948) gives the most generally useful pair of sets of interactions for double confounding in 8 blocks of 8 and 8 blocks of 8 as

*PQRS, RSTU, PQTU, PRT, QST, PSU, and QRU,*  
and *PQRU, PRST, QSTU, PQS, RSU, RQT, and PTU.*

Here the two categories of blocks will correspond to rows and to columns.

If the three factors *P*, *R* and *S* are allocated to denote the 8 levels of a single factor corresponding to 8 solutions (one standard and seven 'unknowns'), *T* to denote dilution, *Q* to denote time of filling (Initial *I* and Final *F*), and *U* to be a dummy factor, then a design is arrived at which is satisfactory in its essentials; for the factors used in calculation of potency, namely the 7 degrees of freedom (*P*, *R*, *S*, *PR*, *RS*, *SP*, *PRS*) corresponding to solutions, and the 1 degree of freedom (*T*) corresponding to dilution, will all be unconfounded. The defect of this design is that 2 out of the 7 degrees of freedom for differences in slope (the interaction between solutions and levels) are confounded, and

therefore it is difficult to carry out an exact sensitive test for parallelism. However, any gross non-parallelism would show itself, and could be checked by a repeat assay of the suspected solution alone against the standard.

Table 1 shows the layout in accordance with the principle of double confounding, with a typical set of results inserted. The design has been randomized with the restriction imposed that in any one row or column the high and low

Table 1. *Selected layout for the assay of seven unknown solutions of penicillin in a 64-hole plate; with a typical set of results (units in millimetres on the enlarger with magnification  $\times 6$ )*

|             |             |             |             |             |             |             |             |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| $6_L^{110}$ | $2_H^{160}$ | $S_L^{120}$ | $1_H^{168}$ | $3_L^{117}$ | $4_H^{155}$ | $5_L^{110}$ | $7_H^{163}$ |
| $S_H^{165}$ | $4_L^{113}$ | $6_H^{150}$ | $7_L^{113}$ | $5_H^{155}$ | $2_L^{110}$ | $3_H^{152}$ | $1_L^{120}$ |
| $5_L^{116}$ | $1_H^{160}$ | $3_L^{110}$ | $2_H^{152}$ | $S_L^{117}$ | $7_H^{155}$ | $6_L^{103}$ | $4_H^{157}$ |
| $3_H^{160}$ | $7_L^{113}$ | $5_H^{150}$ | $4_L^{108}$ | $6_L^{145}$ | $1_L^{114}$ | $S_H^{160}$ | $2_L^{115}$ |
| $1_L^{120}$ | $5_H^{155}$ | $7_L^{113}$ | $6_H^{147}$ | $4_L^{110}$ | $3_H^{150}$ | $2_L^{112}$ | $S_H^{165}$ |
| $7_H^{160}$ | $3_L^{106}$ | $1_H^{159}$ | $S_L^{107}$ | $2_H^{148}$ | $5_L^{100}$ | $4_H^{150}$ | $6_L^{108}$ |
| $2_L^{117}$ | $6_H^{150}$ | $4_L^{110}$ | $5_H^{143}$ | $7_L^{110}$ | $S_H^{155}$ | $1_L^{113}$ | $3_H^{156}$ |
| $4_H^{155}$ | $S_L^{120}$ | $2_H^{160}$ | $3_L^{110}$ | $1_H^{160}$ | $6_L^{102}$ | $7_H^{155}$ | $5_L^{110}$ |

concentrations alternate; one of the reasons for this procedure is that it reduces the risk of any two inhibition zones overlapping. Of the few arrangements possible with this restriction, that selected has the initial and final fillings alternating in columns in sets of four. We justify this departure from pure randomization on the grounds that (a) the mental strain of filling a large number of plates would otherwise be intolerable, and (b) since every plate is different the ground can, as it were, be considered as being randomized underneath the design.

#### *Details of procedure*

To carry out about 70 assays per day 12 plates are required, allowing for a surplus of two in case of accident. For a dozen 150 ml. lots, about 2 l. of assay medium (Evans peptone, 10 g.; Yeastrel, 1.5 g.; Lemco, 3 g.; NaCl, 3.5 g.; Davis agar, 20 g.; distilled water to 1 l., pH 7.0–7.2 before autoclaving) are required.

To obtain it in a perfectly clear state, the medium is first autoclaved in bulk at 20 lb. pressure for 20 min., when some precipitation occurs, filtered through cotton-wool, divided into 150 ml. lots and re-autoclaved at not more than 10–12 lb. pressure for 30 min. to avoid the formation of more precipitate which would affect the clearness of the zones. Sufficient medium for a week's work can be made up at one time. The 12 flasks containing 150 ml. of medium for the day's assay are melted in a steamer, cooled to about 50° and then stood in a water-bath at 45–50° until the temperature is constant, when they are ready for inoculation.

The test organism, *Staphylococcus aureus* 209 P (N.R.R.L./318) is maintained on yeast beef agar slopes (beef extract, 1.5 g.; yeast extract, 2.0 g.; peptone,

6.0 g.; glucose, 1.0 g.; agar, 15 g.; distilled water to 1 l., pH 8.0 before autoclaving at 15 lb. pressure for 20 min.), transferred each evening and incubated overnight at 37°. From this a subculture is made each evening in nutrient broth (peptone, 5.0 g.; yeast extract, 1.5 g.; beef extract, 1.5 g.; NaCl, 3.5 g.;  $K_2HPO_4$ , 3.68 g.;  $KH_2PO_4$ , 1.32 g.; glucose, 1.0 g.; distilled water to 1 l., pH 7.0 before autoclaving at 15 lb. pressure for 20 min.), which is incubated overnight at 37°. The broth is adjusted to an opacity between 1 and 2 on Brown's scale (Burroughs, Wellcome and Co. Ltd. opacity tubes), and 0.3 ml. seeded into 150 ml. of assay medium.

The plates and frames are wiped over with acid ethanol (3 % HCl in 95 % ethanol), which is an adequate means of sterilization, and held at 50° for 1–2 hr., to dry them and to bring them up to the required temperature for pouring. The medium and plates should be at roughly the same temperature to ensure that the medium spreads evenly over the plates before it begins to set. When poured, the plates are placed on a level table, where they may be stacked two or three deep, and left for about an hour. The cavities are then cut with a no. 5 cork borer, previously sterilized by dipping in ethanol and flaming, and the agar plugs picked out with a spear-headed dissecting needle similarly sterilized. A multiple borer may be used which cuts all the cavities at once, but this must be kept well sharpened, otherwise the cavities become irregular.

The penicillin standard is a sodium salt of penicillin G, 1667 units/mg.; a 320 units/ml. solution is made up each day in pH 6.0 buffer (50 ml. 0.1 M- $KH_2PO_4$  + 5.64 ml. 0.1 M-NaOH made up to 100 ml.) and from this the 1.6 units/ml. and 0.4 unit/ml. solutions, used as the  $S_H$  and  $S_L$  respectively, are made up in pH 7.0 buffer (50 ml. 0.1 M- $KH_2PO_4$  + 29.54 ml. 0.1 M-NaOH made up to 100 ml.). The previous day's standard is also put on some plates. It has been found to deteriorate by about 4 % in the 24 hr., and is therefore not suitable for use as a standard; but is a safeguard in case anything should go wrong with the current day's standard.

All fermentation samples are first centrifuged or filtered. Three stages of dilution then follow. The first stage is the same for all samples: 5 ml. are diluted in a 50 ml. volumetric flask with pH 6.0 buffer; this gives a 1 in 10 dilution. The second stage depends on the expected titre of the sample; according to whether this is approximately 160, 240, 320, 480 or 640 units/ml., the dilution used is 5 in 50,  $3\frac{1}{2}$  in 50, 5 in 100,  $3\frac{1}{2}$  in 100 or 5 in 200 respectively in pH 7.0 buffer; this gives the high concentration of the sample, i.e.  $1_H$ ,  $2_H$ , etc. The next stage gives the low concentration,  $1_L$ ,  $2_L$ , etc., and is again the same for all samples. Two ml. of the  $H$  solution are added to 6 ml. of pH 7.0 buffer, previously run into a sample bottle by means of an automatic filler. The  $H$  and  $L$  concentrations of each sample are now in sample bottles ready for filling. All pipettes, sample bottles and centrifuge tubes are sterilized in an oven before use, and volumetric flasks are autoclaved, with the exception of those used for making up standards, which are rinsed with sulphuric acid.

Each operator has her own filler and this must be used throughout a plate, as the fillers vary slightly in the volume they deliver, from about 0.05 to 0.06 ml., and it is important that a constant volume should be delivered each



time. The filler is rinsed with pH 7.0 buffer between all solutions, including the *H* and *L* of the same sample. The time taken to fill a plate is about 10–15 min. and in order to balance the time effect, which is evident after even half this period, two out of the four cavities for each solution are filled first, starting with the  $S_H^I$ ,  $S_L^I$  followed by sample  $1_H^I$ ,  $1_L^I$ , and finishing with sample  $7_H^I$ ,  $7_L^I$ ; the order is then reversed and the second two cavities for each solution are filled, starting with sample  $7_L^I$ ,  $7_H^I$  and returning to sample  $1_L^I$ ,  $1_H^I$  and so back to  $S_L^I$ ,  $S_H^I$ .

The plates are incubated overnight at 37°. It is advisable to distribute the plates on different shelves of the incubator, if possible not more than two deep, because packing increases the amount of condensation, favouring contamination which interferes with the clearness of the zones. The zones are projected for measurement on a screen, magnified six times (Brownlee *et al.* 1948). When finished with, the plates and frames are soaked in disinfectant for at least an hour before washing.

#### *Calculation of the potency ratio*

The usual formula for the potency ratio is  $\text{antilog } dD/B$ , where  $d$  is the logarithm of the dilution ratio, and

$$D = U_H + U_L - S_H - S_L$$

and

$$B = U_H - U_L + S_H - S_L,$$

where  $U_H$ ,  $S_H$  denote the sums of the readings (here four) for the higher concentration of the unknown and standard respectively and  $U_L$ ,  $S_L$  the sums for the two lower concentrations. Both  $U_H - U_L$  and  $S_H - S_L$  are estimates of the slope of the response curve for this particular plate, but in order to get the best estimate we can make use of all seven values of  $U_H - U_L$  along with  $S_H - S_L$ . We should therefore use for  $B'$

$$\frac{1}{4} \left[ (S_H - S_L) + \sum_1^7 (U_H - U_L) \right].$$

#### *The accuracy of the assay in practice*

The analysis of variance of the typical assay shown in Table 1 is given in Table 2. It will be noted from the row and column mean squares, which are large in comparison with the residual mean square, how extremely effective the double confounding arrangement is in removing plate heterogeneity.

Table 2. *Analysis of variance of results in Table 1*

| Source of variance        | Degrees of freedom | Sum of squares | Mean square |
|---------------------------|--------------------|----------------|-------------|
| Rows                      | 7                  | 307.25         | 43.89       |
| Columns                   | 7                  | 415.50         | 59.36       |
| Levels                    | 1                  | 30,537.56      | 30,537.56   |
| Solutions                 | 7                  | 977.50         | 139.64      |
| Solutions $\times$ Levels | 5                  | 19.31          | 3.86        |
| Time                      | 1                  | 42.25          | 42.25       |
| Solutions $\times$ Time   | 5                  | 21.75          | 4.35        |
| Residual                  | 30                 | 155.88         | 5.196       |
| Total                     | 68                 | 32,477.00      |             |

In this particular plate the residual variance  $s^2$  is 5.196. To calculate the internal error of an assay in which the potency of the unknown is close to that of the standard, we use the approximate formula for the standard error  $s_m$  of the logarithm of the potency ratio, which is

$$s_m = \frac{2ds\sqrt{n}}{B'},$$

where  $d$  is the logarithm of the dilution ratio,  $n$  is the number of observations at each of the four points of the assay. Here  $n=4$ ,  $d=0.6021$  and  $B'=349.5$  and hence  $s_m=0.01548$ , with 30 degrees of freedom.

The 95 % fiducial limits for the logarithm of the potency ratio are  $\pm 0.03204$  and thus the limits for the potency ratio are 92.9 and 107.7 %. These limits, however, are based on only the internal error of a plate and we are more interested in external estimates of error, which contain dilution errors, etc. The following experiment was designed to obtain such estimates.

Table 3. *Sets of penicillin assays carried out to determine magnitude of experimental errors*

| Dilution<br>of sample | Operator and Plate no. |     |     |     |     |     |     |     |
|-----------------------|------------------------|-----|-----|-----|-----|-----|-----|-----|
|                       | A                      |     | B   |     | C   |     | D   |     |
|                       | 1                      | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
|                       | units/ml.              |     |     |     |     |     |     |     |
| 1/150                 | 323                    | 294 | 300 | 299 | 300 | 282 | 319 | 317 |
|                       | 370                    | 329 | 312 | 279 | 306 | 285 | 290 | 288 |
| 1/200                 | 299                    | 303 | 316 | 321 | 302 | 287 | 317 | 314 |
|                       | 317                    | 297 | 319 | 303 | 296 | 290 | 314 | 310 |
| 1/300                 | 302                    | 309 | 310 | 307 | 328 | 284 | 312 | 320 |
|                       | 306                    | 317 | 302 | 302 | 323 | 293 | 301 | 301 |

The results of a series of assays to measure the overall error are given in Table 3. Four workers each made up dilutions in duplicate of a standard solution at dilutions of 1/150, 1/200, and 1/300. This gave six solutions for each worker and these were each assayed on two plates. Thus one of the two dilutions at 1/150 made up by operator A gave 323 on the first of her plates and 294 on her second. Her second dilution at 1/150 gave 370 on her first plate and 329 on her second. The seventh position was left blank. The analysis of variance of the results is in Table 4. The variance of a single assay is given by the sum of the components of variance in the last column, viz. 273.47, and the estimated 95 % limits of error for a single assay (including dilution errors) are  $\pm 10.8$  %. In this set of results the errors in repeat dilutions are rather large; they may have arisen from inadequate shaking of the flasks.

This standard of accuracy can be attained in practice. Over several days 61 fermenter vessels were sampled and assayed in duplicate. The range between duplicates corresponded to 95 % error limits of  $\pm 9.6$  %. Should greater accuracy than this be required, it can be readily obtained by replication, the error limits of course being inversely proportional to the square root of the

number of replications. It is obvious that the replication should take place on different plates. Sevenfold replication, equivalent on the average to one unknown per plate, would give 95 % fiducial limits of 3.65 %.

Table 4. *Analysis of variance of results in Table 3*

| Source of variance                         | Degrees of freedom | Sum of squares | Mean square | Component of variance |
|--|--------------------|----------------|-------------|-----------------------|
| Operators                                  | 3                  | 1,569.56       | 523.187     | —                     |
| Dilutions                                  | 2                  | 18.00          | 9.000       | —                     |
| Operators $\times$ Dilutions               | 6                  | 2,696.50       | 449.417     | —                     |
| Plates within Operators                    | 4                  | 2,074.42       | 518.605     | 55.827*               |
| Plates within Operators $\times$ Dilutions | 8                  | 1,508.83       | 188.604     | 67.625                |
| Between repeat Dilutions within Operators  | 12                 | 2,972.25       | 247.687     | 97.167                |
| Residual                                   | 12                 | 640.25         | 53.354      | 53.354                |
| Total                                      | 47                 | 11,479.81      | —           | —                     |

\* In calculating this component of variance the Operators Mean Square has been pooled with that for Plates within Operators.

To compare the relative efficiency of these large plates with that of Petri dishes is not easy. Considering merely the number of holes to be cut, filled and measured, this sevenfold replication is equivalent to sixteen Petri dishes per unknown, but with Petri dishes we have never been able to obtain 95 % fiducial limits anything like as low as 3.65 %. A single plate is also probably more easily handled, poured, washed, etc., than sixteen Petri dishes. It has been our experience that four operators can deal with 10 plates per day, i.e. testing 70 unknown solutions.

It will be noted that the error between assays on the same plate is less than the error between assays on different plates, and therefore when one is engaged in experiments comparing different treatments, and not immediately concerned with the absolute value, it is advantageous to arrange the treatments so that they occur in blocks of seven, if necessary using the balanced incomplete block designs, a table of which is given by Fisher & Yates (1943).

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# The Relationship of the Aluminium Phosphate Precipitation of Organisms of *Haemophilus pertussis* strains to their other Biological Properties

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**SUMMARY:** Of 46 strains of *Haemophilus pertussis*, 34 recently isolated strains were all agglutinated by Phase 1 antiserum. Of 12 laboratory strains, 5 had lost their agglutinability. The agglutinable strains were precipitated by aluminium phosphate, and lysed by sodium hydroxide or 10% sodium desoxycholate and were virulent to mice. The virulent strains, which were precipitable by aluminium phosphate, produced toxic substances in a fluid culture. Precipitation by aluminium phosphate provides a quick method for distinguishing virulent and avirulent strains of *H. pertussis*.

Suspensions of strains of *Haemophilus pertussis* that are agglutinated by a Phase 1 antiserum have been found also to be completely precipitated with aluminium phosphate, whereas strains that become non-agglutinable by the Phase 1 antiserum also lose this precipitability (Ungar & Muggleton, 1948). The object of the present communication is to show the relationship between precipitability, agglutinability and certain other biological properties of different strains of *H. pertussis*, either freshly isolated from recent cases of pertussis or maintained in the laboratory for some time.

It has long been known that strains of *H. pertussis* maintained in artificial culture can pass through a series of serologically differentiable phases. In our study no attempt was made to classify the strains used into phases, except that we assigned to Phase 1 all strains that were agglutinated to a high titre (over 1/8000) with a rabbit antiserum prepared by inoculation with a freshly isolated strain.

Altogether we tested 46 strains, of which 34 were received, immediately after isolation, from the Central Public Health Laboratory of the Public Health Laboratory Service or from the North Western Group Laboratory of the L.C.C., and one from Prof. G. A. H. Buttle of the School of Pharmacy, University of London. The remainder were stock strains maintained in this laboratory for various periods; of these, six had been repeatedly subcultured on Bordet-Gengou medium and were no longer agglutinable with the Phase 1 antiserum and the rest had been freeze-dried and stored. All the strains were kept freeze-dried during the investigation and frequent culture from the freeze-dried state was made to ensure that the strains used in the tests remained constant.

## *Aluminium phosphate precipitation of the strains*

A standard method of testing the precipitability of the strains was used throughout. The suspensions from 48 hr. Bordet-Gengou cultures were washed twice, resuspended in saline containing 0.2% formaldehyde, to give a concentration of *c.*  $10^{10}$  organisms/ml., and held at room temperature overnight.

To 4 ml. of suspension in a test-tube 0.8 ml. of 5 %  $\text{Na}_2\text{HPO}_4$  was added, followed by 1 ml. of 2 % potassium alum, which was added slowly with constant stirring. The mixture was vigorously agitated for two minutes. After standing for 30 min. the floccules had settled out, leaving a water-clear supernatant fluid when precipitation was complete. With strains that were not precipitated the supernatant fluid remained turbid and by comparing it turbidimetrically with a control suspension the percentage of precipitation could be estimated. Precipitation was usually performed at pH 6.5. The standardized technique for aluminium phosphate precipitation of the suspensions was devised on the basis of tests in which the concentrations of the phosphate and alum solutions were varied. These tests fixed the minimum amount of adsorbent needed to precipitate all the organisms of a suspension of an average agglutinable strain; for the standard technique double this amount was used. Adsorption of the organisms was found to take place immediately the potassium alum solution was added at room temperature; the two minutes' shaking gave the bacterial cells every chance to come into contact with the phosphate.

#### *The agglutinability of the strains*

The strains were tested for agglutinability by using a twice washed, 0.2 % formaldehyde-killed suspension containing  $2000 \times 10^6$  organisms/ml. (Brown's opacity tubes manufactured by Burroughs, Wellcome & Co., London). Equal volumes (0.5 ml.) of serial dilutions of Phase 1 rabbit antiserum and suspensions were mixed in Dreyer's tubes and incubated for 2 hr. at  $37^\circ$  in a water-bath. The same batch of serum was used throughout the tests and the agglutinability of the strain was recorded as the greatest dilution of serum that would just cause granular agglutination visible to the naked eye.

All the 46 strains of *H. pertussis* were tested for precipitability and agglutinability in this way, with results recorded in Table 1.

Table 1. *Relationship between agglutinability of Haemophilus pertussis strains with Phase 1 antiserum and precipitability with aluminium phosphate*

|                                       | Number of strains tested and origin |                            |                            |
|---------------------------------------|-------------------------------------|----------------------------|----------------------------|
|                                       | 5 lab. stock                        | 7 lab. stock               | 34 freshly isolated        |
| Agglutination titre<br>Phase 1 serum  | 1/ < 1000                           | 1/12,000 to 1/16,000       | 1/8,000 to 1/30,000        |
| Behaviour with<br>aluminium phosphate | Not precipitated                    | Completely<br>precipitated | Completely<br>precipitated |

Strains agglutinated by serum dilutions of 1/8000 or more were completely precipitated by the aluminium phosphate. One of the strains (83) grown from the freeze-dried state could be induced to change phase relatively quickly by about six subcultures only, on Bordet-Gengou medium. The strain was tested at each subculture and it was found that agglutinability and precipitability diminished in parallel after the 5th to the 8th subculture.

Suspensions of *H. pertussis* killed and stored in 0.5 % aqueous phenol lose their agglutinability on storage in the refrigerator (Ungar, 1947). It was found,

however, that suspensions so treated remained precipitable with aluminium phosphate; this suggests that the loss of agglutinability by spontaneous change of phase on repeated subculture differs in mechanism from that caused by treatment with phenol.

#### *Susceptibility of strains to other precipitants*

Various substances other than alumina and aluminium phosphate precipitate the suspensions. Cadmium phosphate was tested by adding 0.3 ml. of 5 % disodium phosphate and 1 ml. of 2 % cadmium chloride to 4 ml. of suspension. Crystalline magnesium ammonium phosphate was tested by adding 0.3 ml. of 5 % disodium phosphate and 1 ml. of 2 % magnesium phosphate followed by 0.01 ml. of ammonia (sp. gr. 0.880) to 4 ml. of suspension. The percentage of the suspended organisms precipitated by these four adsorbents, using seven strains, and the agglutination titres are tabulated in Table 2.

Table 2. *Comparison of agglutinability of seven strains of Haemophilus pertussis with their precipitability by alumina, aluminium phosphate, cadmium phosphate and magnesium ammonium phosphate*

| Strain | Agglutination titre | Percentage precipitability by |                     |                   |                              |
|--------|---------------------|-------------------------------|---------------------|-------------------|------------------------------|
|        |                     | Alumina                       | Aluminium phosphate | Cadmium phosphate | Magnesium ammonium phosphate |
| 82     | 1/ > 16,000         | 100                           | 100                 | 100               | 90                           |
| 83     | 1/ > 16,000         | 100                           | 100                 | 100               | 90                           |
| 104    | 1/ > 16,000         | 100                           | 100                 | 100               | 85                           |
| 102    | 1/ < 500            | 100                           | 20                  | 10                | 0                            |
| 105    | 1/ 1,000            | 80                            | 50                  | 85                | 75                           |
| 167    | 1/ < 500            | 80                            | 0                   | 0                 | 0                            |
| 154    | 1/ < 500            | 90                            | 0                   | 0                 | 0                            |

All four adsorbents behave similarly in that the agglutinable strains are precipitated to a greater extent than the non-agglutinable, the precipitation being complete with each substance except the magnesium salt. It is likely that the mechanism of adsorption is the same for all of them, the degree of adsorption being dependent on the adsorbent used.

Tests on representative strains were designed to elucidate the difference between the two types of organism.

*Morphology and staining.* Gram- and Giemsa-stained films of the strains used showed that the non-agglutinable organisms were smaller and more coccoid in shape than the agglutinable; they tended to be Gram-positive and also had a slightly greater affinity for basic dyes, which suggested a greater potential acidity in these organisms than in the non-agglutinable. The pH of suspensions that took up stain in this way was accordingly measured by means of a glass electrode; no appreciable difference in pH was observed between suspensions of the two types. Measurements of this kind probably give no indication of the potential acidity of the cells, which is what determines their staining affinities. It occurred to us, however, that there might be different pH

optima for precipitation of the two types of suspension with aluminium phosphate. We tested the precipitability of suspensions adjusted to various pH values with hydrochloric acid and sodium hydroxide over the range 4.0–8.0. The pH of the suspension made little difference; non-agglutinable strains were not precipitated over this pH range, whereas agglutinable strains remained precipitable over the whole of it. Below pH 4.0 the aluminium phosphate became increasingly soluble and even agglutinable strains were not precipitated.

The strains were examined at several stages of growth by a modification of Fleming's capsule stain (Jeffrey, 1948), but there was no marked difference between the non-agglutinable and the agglutinable organisms grown for 48 or 72 hr. Capsules were demonstrable in both types of organism and varied slightly in depth of staining and thickness according to the age of the culture.

*Growth characteristics.* It is known that freshly isolated strains will grow only on Bordet-Gengou medium containing about 25 % blood. By repeated subculture we diminished the percentage of blood required to support growth until the strains became non-agglutinable, when they grew sparingly on ordinary digest agar without added blood. In a liquid partly defined culture medium (Cohen & Wheeler, 1946) the non-agglutinable strains also grew more rapidly, producing a uniform turbidity in the medium, instead of the stringy surface pellicle seen in cultures of freshly isolated, agglutinable strains.

*Chemical reactions.* During the investigation of the effect of pH on precipitation with aluminium phosphate, it was observed that the addition of a slight excess of sodium hydroxide solution to a suspension of agglutinable organisms caused a rapid lysis of the bacteria, accompanied by an increase in the viscosity of the fluid. This did not occur with a non-agglutinable suspension, and we therefore investigated the solubility of suspensions of different strains. To 4 ml. of a standard suspension containing  $20,000 \times 10^6$  organisms/ml. in test-tubes, 1 ml. of N-NaOH was added. After 30 min. the precipitable organisms were invariably lysed, whereas non-precipitable ones were not. A similar result was obtained with the alkaline 10 % sodium desoxycholate solution normally used for investigating the bile solubility of *Streptococcus pneumoniae*. The solubility of the alum-precipitable strains suggested that this phenomenon might be similar to that encountered in pneumococci, in which a polysaccharide is an essential component of the capsule. Concentrated suspensions of both types of organisms were positive when tested for the presence of polysaccharides by Molisch's reagent.

Suspensions of agglutinable and non-agglutinable organisms were examined electrophoretically after dialysis against a phosphate buffer at pH 6.5; the value of  $\mu$  at 4° for 2 days was 0.2. The organisms in both suspensions migrated towards the anode, indicating that they are negatively charged at this pH, but there was no clear difference between the two types of suspension.

#### *Comparison of agglutinability and precipitability with virulence in mice*

An attempt was made to associate agglutinability and aluminium phosphate precipitability with the virulence of the strains to mice after intranasal instillation (Burnet & Timmins, 1937). The 48 hr. growth on Bordet-Gengou

medium was scraped off, washed twice in saline and instilled (0.1 ml. containing  $200 \times 10^6$  organisms) into the nostrils of six albino mice (weight 16–18 g.) under ether-chloroform anaesthesia; the strain of animals was descended by close inbreeding from pure-line Strong A 2 ancestors. Virulence was assessed according to the number of mice remaining alive on the 10th day. At the time of infection, organisms from the same cultures were tested for agglutinability, aluminium phosphate precipitability and bile- and soda-solubility. The results summarized in Table 3 indicate a close correlation between precipitability, agglutinability and the bile- and soda-solubility of virulent and avirulent strains and that aluminium phosphate precipitation provides a simple method for distinguishing the two types.

Table 3. *Relationship between agglutinability, precipitation with aluminium phosphate, virulence in mice and NaOH and bile solubility*

| Strain no. | Agglutination titre<br>Phase 1 serum | $\text{AlPO}_4$ precipitation | Virulence in mice.<br>No. surviving group after 10 days | Solubility |      |
|------------|--------------------------------------|-------------------------------|---|------------|------|
|            |                                      |                               |   | NaOH       | Bile |
| 82         | 1/16,000                             | ++                            | 1/6   | ++         | ++   |
| 83         | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 102        | 1/1,000                              | 50%                           | 6/6   | 0          | 0    |
| 104        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 105        | 1/ < 1,000                           | 0                             | 3/6   | 0          | 0    |
| 146        | 1/ < 1,000                           | 0                             | 6/6   | 0          | 0    |
| 154        | 1/ < 1,000                           | 0                             | 6/6   | 0          | 0    |
| 167        | 1/ < 1,000                           | 0                             | 6/6   | 0          | 0    |
| 191        | 1/20,000                             | ++                            | 0/6   | ++         | ++   |
| 192        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 198        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 199        | 1/8,000                              | ++                            | 2/6   | ++         | ++   |
| 200        | 1/12,000                             | ++                            | 0/6   | ++         | ++   |
| 201        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 202        | 1/8,000                              | ++                            | 3/6   | ++         | ++   |
| 203        | 1/4,000                              | ++                            | 3/6   | ++         | ++   |
| 204        | 1/8,000                              | ++                            | 0/6   | ++         | ++   |
| 205        | 1/30,000                             | ++                            | 0/6   | ++         | ++   |
| 206        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 'N'        | 1/30,000                             | ++                            | 1/6   | ++         | ++   |
| 'T'        | 1/16,000                             | ++                            | 0/6   | ++         | ++   |
| 'O'        | 1/4,000                              | ++                            | 0/6   | ++         | ++   |

++ = Complete precipitation or solubility.

The intracerebral route of infection (Kendrick, Eldering, Dixon & Misver, 1947) was unsuitable owing to the great differences found between the minimal lethal doses of various strains; moreover only with a few strains have we obtained consistent results by this method.

#### *'Toxin' produced during growth*

We compared the production of toxic substances during growth of the agglutinable and the non-agglutinable strains in the liquid partly defined medium of Cohen & Wheeler (1946). A number of strains of both types were grown for 12 days at 37° in 10 oz. medical flat bottles containing 100 ml. of culture. On



the 12th day 50 ml. quantities of all the cultures were centrifuged for 2 hr. at c. 3500 r.p.m., and the supernatant fluids removed. One-fifth ml. of dilutions of 1/25 and 1/100 in saline were injected intradermally into the depilated flank of a rabbit, and the toxic reaction was recorded after 48 hr. The centrifuged organisms were resuspended in 0.2 % formol saline and tested for agglutinability. Our results (Table 4) confirmed the observations of Wood (1940), who used serum broth medium and tested the toxicity of the culture fluid by intraperitoneal injection in mice, in that most freshly isolated strains in Phase 1 produce a potent 'toxin' during their growth in liquid medium.

Table 4. *Relationship between agglutinability and 'toxin' production in liquid semi-defined medium*

| Strain no. | Agglutination<br>titre of organisms | Toxic reaction in rabbit skin |             |
|------------|-------------------------------------|-------------------------------|-------------|
|            |                                     | 1/25 toxin                    | 1/100 toxin |
| 82         | 1/10,000                            | ++                            | +           |
| 83         | 1/10,000                            | +++                           | ++          |
| 102/A      | 1/10,000                            | +                             | +           |
| 104        | 1/10,000                            | +++                           | ++          |
| 105*       | 1/ < 500                            | +                             | +           |
| 146        | 1/ < 500                            | 0                             | 0           |
| 154        | 1/ < 500                            | 0                             | 0           |
| 167        | 1/ < 500                            | 0                             | 0           |
| 191        | 1/10,000                            | ++                            | 0           |
| 192        | 1/10,000                            | ++                            | ++          |
| 196        | 1/5,000                             | +                             | 0           |
| 199        | 1/10,000                            | +                             | +           |
| 203        | 1/10,000                            | +++                           | ++          |
| 204        | 1/5,000                             | +                             | +           |
| 209        | 1/10,000                            | +++                           | +++         |
| 210        | 1/10,000                            | ++                            | ++          |

+++ = Dermonecrotic reaction and haemorrhage c. 4 cm. diameter.

++ = Dermonecrotic reaction and haemorrhage c. 2 cm. diameter.

+

0 = No reaction.

\* Strain 105 is exceptional in that, besides forming a toxic substance, it is partly virulent in mice (see Table 3).

The non-agglutinable strains, with the exception of strain 105, did not produce a potent toxin. An investigation of the toxins produced by some of the Phase 1 strains confirmed Wood's observations that the culture fluids are detoxified by Seitz filtration, and by treatment with 0.2 % formaldehyde for two days, and by mild heat. We found further that the toxic solution was detoxified on passing through a diatomaceous earth candle or a sterilizing (No. 5) sintered glass filter, as well as by prolonged high-speed centrifugation (with precautions against a rise in temperature). The toxic antigenic component can also be precipitated with aluminium phosphate in the same way as a bacterial suspension (Ungar & Muggleton, 1948). These observations lead us to the conclusion that the toxin is probably a suspension of very fine particles rather than a true solution and is the result of disintegration of dead bacterial cells during growth of the culture. This may be related to the fact that the non-agglutinable strains, which do not

produce a toxic culture fluid, may be more resistant to disintegration, just as they are more resistant to the action of sodium hydroxide and bile salts. Alternatively the change of phase may be accompanied by cessation of toxin production. Table 5 summarizes the differences between the two types of strain.

Table 5. *Summary of differences between strains of Haemophilus pertussis*

| Property                               | Agglutinable strain<br>(Titre 1/> 8000 with<br>Phase 1 serum) | Non-agglutinable strain<br>(Titre 1/< 500 with<br>Phase 1 serum)   |
|--|---|--|
| Precipitation with aluminium phosphate | Complete precipitation  | No precipitation   |
| Morphology                             | Short Gram-positive bacillus, about $1.5\ \mu$                | Shorter and smaller than agglutinable organisms, about $1\ \mu$ , and tends occasionally to be Gram-positive |
| Capsulation                            | Capsulated (wide zone noticed after 24 hr.)                   | Capsulated   |
| Solubility in NaOH and bile salt       | Completely soluble  | Insoluble  |
| Virulence in mice                      | Mostly virulent   | Avirulent  |
| Endotoxin production in liquid medium  | Mostly toxin producing  | Non-toxic  |

## DISCUSSION

After isolation from the human host, strains of *Haemophilus pertussis* undergo changes of phase on repeated subcultivation and lose their ability to be agglutinated by Phase 1 antiserum. Accompanying this loss of agglutinability is some change that makes the cell less susceptible to adsorption by aluminium phosphate and consequently less precipitable. The means by which bacterial cells are adsorbed on such insoluble compounds is not properly understood. It is possibly due to differences in charge on cells and adsorbent, but if this be so, the process is strangely unaffected by change of pH. Cells that have lost their agglutinability by Phase 1 antiserum and are no longer susceptible to the dissolving action of sodium hydroxide or bile salt still have capsules demonstrable in stained preparations. This suggests a condition similar to that encountered in the streptococcus group, in which the bile solubility of *Streptococcus pneumoniae* is associated with an antigenically active polysaccharide capsule. The fact that the loss of agglutinability with Phase 1 serum shows fairly close correlation with the loss of virulence to mice infected by the intranasal route, and the absence of endotoxin when grown in simplified media, are noteworthy. The changes that take place on repeated subculture, resulting in a much more rapid growth and simpler growth requirements, are certainly accompanied by a 'deterioration' of the strains. It is well known that among some of the pathogenic bacteria the prevalence of rough dissociants of laboratory strains is accompanied by loss of virulence and toxigenicity, and this also seems to occur with *Haemophilus pertussis* strains.

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## Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

### 4. The Partially Acid-fast Proactinomycetes

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**SUMMARY:** About three hundred strains of proactinomycetes were isolated from Rothamsted soils and examined together with certain strains from the National Collection of Type Cultures and other sources. Although little more than 9% were partially acid-fast on immediate isolation, subcultivation on rich media such as milk or nutrient glucose broth increased the percentage to 31%. The strains showed a range of features, from the soft mycobacterial type of growth with transient vegetative mycelium and very sparse aerial mycelium, if any, to the harder, more actinomycete-like variety. Of the acid-fast species *Proactinomyces opacus*, *Proactinomyces salmonicolor*, and *Proactinomyces paraffinae* predominated. In all, ninety-two strains were observed continuously for nearly two years on a variety of complex and simple media and were stained at frequent intervals. Acid-fast cell elements occurred more often in complex protein- and fat-containing media, and in chemically defined media containing paraffin or large quantities of glycerol. They varied in shape and size according to the species and the age and quality of the growth. It is thought that differences of permeability of the cytoplasmic membrane in different environments may account for these variations. No evidence was obtained of any 'resting spores' or 'chlamydo spores' in the vegetative mycelium. Since there are no true spores in the aerial mycelium when it is present, the proactinomycetes in general may be regarded as asporogenous.

The proactinomycetes (Jensen, 1931, 1932), also known as *Actinomyces* Groups IIa and IIb (Ørskov, 1923) and *Nocardia* (Waksman & Henrici, 1943; Bergey, 1948), are distinguished morphologically from the true actinomycetes (*Streptomyces* Waksman & Henrici) by (1) the transient nature of the vegetative mycelium, which may break up into rods and cocci after 1-3 days, and so produce a soft growth resembling that of mycobacteria; and (2) the infrequent development of an aerial mycelium, which may fragment, but does not divide evenly into spores, and which is often only of microscopical dimensions.

Most workers agree that the sparse, often rudimentary, aerial mycelium cannot be regarded as a sporogenous phase. Yet genuine sporing actinomycetes can at times produce asporogenous variants, which develop at the most only a few undivided aerial hyphae exactly similar to those of proactinomycetes (Appleby, 1947; Erikson, 1948). It is not impossible that there should be conditions under which proactinomycetes are stimulated to greater aerial growth, and that the cell contents of some of the aerial filaments should divide and behave as happens in the corresponding hyphae of actinomycetes. It is, however, in the very variable shape and size of the cell elements of the substratum or vegetative growth on different media that the great plasticity of this group is expressed. In the older medical literature and in the more recent work of Krassilnikov (1934) on soil organisms allied to mycobacteria, there is

frequent mention of peculiar swollen spore forms such as 'chlamydospores', 'resting spores', 'cystites', 'arthrospores', which can be distinguished by their size and staining properties.

The property of acid-fastness has been reported for many members of this group. Together with angular division, it is one of the characteristics which allies them with the mycobacteria. Jensen (1931), when proposing the species *Proactinomyces paraffinae*, described certain branches of the vegetative mycelium as dividing up into acid-fast, spore-like bodies. On the other hand, it has long been known that the conidiospores of the aerial mycelium of certain species of actinomycetes are acid-fast (Lieske, 1921; Erikson, 1935; Basu, 1937). Bacterial endospores are also acid-fast. In this study a search was made in a large range of partially acid-fast proactinomycetes to determine: (a) which cell elements under a variety of cultural conditions are resistant to acid decolorization, using the method of Umbreit (1939); (b) whether they occur consistently and are capable of being interpreted as a sporogenous phase; (c) what factors favour the development of aerial mycelium.

#### *Source of the strains studied*

Conn & Dimmick (1947) state that 'partially acid-fast organisms, apparently related to mycobacteria, do occur in soil; but... they do not seem to make up part of the predominant soil flora'. Jensen (1931), who used a complex casein agar medium for isolation, also considered that they were rare, and that it was necessary to make a special search for them or to use selective methods such as adding paraffin to the soil. Recent work in this department, following that of Gray & Thornton (1928), suggests that they are fairly widely distributed. Thus, of eighteen *Proactinomyces* colonies picked at random from soil-extract agar plates poured by a colleague in the routine plating of Rothamsted soils, twelve were found to be partly acid-fast when grown on nutrient glucose broth. That they can be easily overlooked is shown by the following experiment. Three soil plots were sampled, and all presumed proactinomycete strains were immediately tested for acid-fastness in the first subculture on semisolid soil-extract agar. The yields were: Plot A, 26 strains, 5 of them acid-fast; Plot B, 37 strains, 2 acid-fast; Plot C, 28 strains, 1 acid-fast. But when these same ninety-one strains were subcultured three to four times on nutrient glucose broth or in milk and re-tested, the numbers acid-fast were: Plot A, 26 strains, 14 acid-fast; Plot B, 37 strains, 11 acid-fast; Plot C, 28 strains, 12 acid-fast. These findings were confirmed at intervals, with one or two exceptions, and eventually 33 of the 91 strains proved to be partially acid-fast on some medium. This apparent enhancement of acid-fastness after the first isolation from soil is contrary to the usual decrease in such staining properties in pathogenic organisms of this group obtained from animal material. In this last case, however, they are generally grown on rich media from the beginning.

Other experiments yielded similar results, and in all, during a period of nearly two years, about 800 proactinomycete strains were examined. In addition, a few strains were received through the kindness of Dr Turfitt (from soils) and of Dr Sharp (from human pathological material). Some National

Collection Type Cultures were included for comparison. From this total 82 strains were selected as showing acid-fast elements on some media. The majority could be assigned to known species listed in Bergey (1948) under the generic name *Nocardia*, and are as follows (the older nomenclature being retained for the present).

*Proactinomyces minimus* Jensen, 4 strains; *Proactinomyces opacus* den Dooren de Jong, 12 strains; *Proactinomyces polychromogenes* Vallee, 2 strains; *Proactinomyces paraffinae* Jensen, 14 strains; *Proactinomyces salmonicolor* den Dooren de Jong, 18 strains; *Proactinomyces coeliacus* Gray & Thornton, 4 strains; *Proactinomyces ruber* Krassilnikov, 3 strains; 18 unidentified.

National Collection Type Cultures: No. 576, *Actinomyces luteus* Christopherson & Archibald; No. 659, *Actinomyces caprae* Silberschmidt; No. 6115, *Actinomyces rhodii* Erikson; No. 3488, *Proactinomyces paraffinae* Jensen; No. 3486, *Proactinomyces polychromogenes* Vallee; No. 2568, *Mycobacterium convolutum* Gray & Thornton; No. 2569, *Mycobacterium erythropolis* Gray & Thornton; No. 2566, *Mycobacterium crystallophagum* Gray & Thornton; No. 2563, *Mycobacterium agreste* Gray & Thornton; No. 2571, *Mycobacterium actinomorphum* Gray & Thornton; No. 525, *Mycobacterium phlei* Lehmann & Neumann.

Unidentified mycobacterium 'Hewison' (human source).

Since *Proactinomyces opacus*, *Proactinomyces salmonicolor*, and *Proactinomyces paraffinae* predominated in the soil strains, were clearly marked types, and together covered almost the complete range of the features characteristic of the genus, they were studied in detail.

#### *Proactinomyces opacus*

(a) *Cultural characters.* A soft cream to pink growth on nutrient agars. Most strains were of the *crystallophagum* type, moister and softer, as described by Jensen, and not so filamentous as his *opacus* strains. There is little doubt that they should be classed together. On chemically defined agar media such as starch-nitrate, ammonium lactate, and Oxford's (1946) ammonium acetate medium, growth was colourless and thin, and produced an initial mycelium the filaments of which were more or less quickly divided into short rods. The addition of 0.01 %  $\text{MnSO}_4$  to Czapek's sucrose-nitrate agar usually stimulated the production of the pale pink pigment in the very moist, almost mucoid type of growth which was characteristic of these strains.

(b) *Incidence of acid-fast elements.* Each strain was tested daily for a week, and thereafter every week for three months, with the following results.

Nutrient glucose broth: small mycelia and branching filaments, variably acid-fast, up to the fourth day; from the fourth to the fourteenth day filaments scarce, generally not acid-fast, and short beaded rods, partly acid-fast, predominant; after the second week more acid-fast rods in the cream surface scum than in the copious bottom deposit; at three months very short rods, sometimes coccoid, almost all non-acid-fast in both surface and bottom growth; broth occasionally turbid in the first week, afterwards clear.

Milk: short rods, beaded, mostly not acid-fast, some branching filaments up to the third day; from the fourth to the fourteenth day gradual increase in length of filaments, branching mycelia, and general acid-fastness; at six weeks

surface growth shows mostly short rods and cocci which are somewhat more acid-fast than the comparable elements in the bottom growth; mostly non-acid-fast at three months.

Czapek salts (nitrate) + liquid paraffin: the branching filaments are more often not acid-fast and the short rods positive and variable.

Ammonium phosphate, Czapek salts + solid paraffin: mostly short rods, positive.

Czapek salts (nitrate) + increasing amounts of glycerol: short rods negative or variable up to 2.5 % glycerol; from 5 to 12.5 % coccoid rods, often in chains, constantly acid-fast.

Czapek salts (nitrate) + 1 % various carbon sources (sucrose, glucose, galactose, maltose, lactose, xylose, sorbitol, dulcitol): few branching filaments, mostly short rods, negative or occasionally variable, whether growth is good as in galactose, sorbitol, glucose, sucrose, maltose, or poor as in lactose, xylose, dulcitol; no acid produced.

Czapek salts (sucrose) + 0.1 % various nitrogen sources (sodium nitrate, ammonium phosphate, ammonium lactate, alanine, glycine, urea): similar, occasional branching filaments, mainly short rods, non-acid-fast, whether growth is poor (urea) or fair to good (all others).

Thus, acid-fast cell elements predominated at the period of maximum growth in a free air supply in complex media; in a chemically defined medium they could be found only when substances such as paraffin or large quantities of glycerol were added; in all instances the elements which retained the stain were normal vegetative cells—branching filaments and the short rods into which they divide as the result of population pressure; cocci or large swollen cells were very rarely seen.

(c) *Development of aerial mycelium.* The short simple aerial filaments, which on media like glucose, asparagine and starch-tryptone agar appear on the first to the third days then quickly wither away again, have been admirably depicted by Jensen (1981). I agree with him in finding no division into spores, and no difference in staining properties. Most of these strains produced a very thin dry growth on Oxford's ammonium acetate agar, which tended to inhibit vegetative division so that the mycelia remained intact for 1–3 weeks and gave rise to aerial filaments that were relatively long and sometimes branched but not divided. When a cover-slip was pressed over these growths, it was impossible to distinguish between substratum and aerial mycelium; there was no difference in refractility, density of protoplasm, width of filament, or thickness of cell wall.

#### *Proactinomyces salmonicolor*

(a) *Cultural characters.* A rich salmon pink to yellow-pigmented soft growth on nutrient agars, and a more pasty and sometimes crumbly consistency compared with *P. opacus*. Growth fair to good and usually coloured on the simpler media such as starch-tryptone and ammonium lactate agars; particularly good and characteristic on Czapek's sucrose nitrate agar + 0.01 %  $\text{MnSO}_4$ , becoming somewhat dry and considerably convoluted. One strain immediately after isolation produced a darkening of the medium, but this property was lost

on subsequent cultivation. The cells on this medium are remarkably broad, highly refractile, and intensely Gram-positive. The development of large swollen, spherical, pear-shaped and other irregular elements can be seen in hanging-drop broth cultures. They are to be found towards the centre of the drop after the initial division of the minute mycelium. At the margins of the drop where the film of liquid has spread thinly on the surface of the slip, the filaments tend to be longer and to spread in parallel bundles. The production of 'cystites' is used as a diagnostic criterion in Bergey's (1948) classification.

(b) *Incidence of acid-fast elements.* Nutrient glucose broth: minute mycelia and branching filaments up to 24 hr., mostly negative; few long filaments on the second day, generally acid-fast; from the third to the fourteenth day rods, often showing bipolar beading, and cocci, variable and negative; thereafter gradual loss of acid-fastness in beaded rods and chains of cocci; broth generally clear; no significant difference in staining reactions of top and bottom growth.

Milk: up to the third day generally short branching filaments and rods, mostly negative; from fourth to fourteenth day increase in length of filaments which are usually strongly beaded and acid-fast; no difference in bottom growth; later, rods and cocci, mainly negative.

Czapek solutions + liquid or solid paraffin: bipolar rods, long filaments, and chains of cocci, all usually acid-fast.

Czapek salts + increasing amounts of glycerol: filaments and rods, mainly negative up to 2.5 %; variable at 5 %; mostly rods, constantly acid-fast, from 7.5 to 12.5 %.

Chemically-defined solutions with various carbon and nitrogen sources: as for *P. opacus*, with the exception that cocci are generally produced; usually non-acid-fast; but the addition of a small quantity of  $\text{MnSO}_4$  (0.01 %) to the sucrose nitrate medium resulted in numbers of beaded filaments, rods and cocci showing partial acid-fastness; four strains grew poorly in *m*-cresol as carbon source and were all acid-fast.

Here, although the developmental picture is of greater complexity than in the preceding species, acid-fastness is not restricted to any special shape of cell. The large, spherical, or irregularly swollen 'cystites' are relatively few, appear in the early stages of growth, and are seldom acid-fast. In the strongly beaded rods and filaments which are typical of this species in all cultures, it is generally the cell walls that retain the basic fuchsin for the longest periods, while the condensed beads of cytoplasm take the counterstain. The enhancing effect of such additions as paraffin and glycerol is not permanent; subculture from these media to starch-tryptone agar, on which the growth is usually non-acid-fast, yields cells that do not resist acid decolorization.

(c) *Development of aerial mycelium.* A few short, undivided aerial filaments appear occasionally on starch-tryptone, and more often and more persistently on ammonium acetate agar. On one-month-old Czapek sucrose nitrate plates the rather dry, convoluted pink growth of some strains developed a thin white frosting of aerial mycelium. Certain of the individual threads later produced septa at irregular intervals, but no differences in permeability or resistance to dyes could be established for these aerial cells.



*Proactinomyces paraffinae*

(a) *Cultural characters.* A hard, firm, yellowish growth with a visible amount of white aerial mycelium on most nutrient agars. On starch nitrate and glucose ammonium phosphate agars growth is thinner, and frequent bulbous swellings at the ends of the long initial filaments as described by Jensen can be seen. Angular branching and division into chains of cocci both occur within 2–7 days. On poorer media such as ammonium lactate and ammonium acetate agars, the original minute mycelium divides almost completely by the characteristic ‘slipping’ method into an assemblage of short rods, and only at the margins of the colony are longer filaments present, giving a rhizoid appearance to the growth. The production of chains of acid-fast cocci in the vegetative mycelium is claimed as a specific character.

(b) *Incidence of acid-fast elements.* Nutrient glucose broth: the general picture is similar to that of *P. salmonicolor*, except that acid-fastness is more infrequent; in the early stages minute mycelia, branching filaments and rods may all show acid-fastness; gradually cocci become predominant, but like the former cell elements they are variably acid-fast.

Milk: as in the other two species, a gradual increase in positive staining reactions and filament length, followed by loss of acid-fastness and division into rods and coccoid chains.

Chemically defined solutions + paraffin: this species grows exceptionally well, as its name implies, but the enhancement of acid-fast staining is not so marked. All the cell elements, branching filaments, rods, and cocci, have been noted as variably acid-fast.

Chemically defined solutions + increasing amounts of glycerol: acid-fastness is general at the higher levels, 7·5–12·5 %, and the predominant picture is of short rods.

Chemically defined solutions + various sources of carbon and nitrogen: most strains are negative throughout, but the addition of 0·01 %  $\text{MnSO}_4$  to Czapek’s sucrose nitrate causes certain strains to show an appreciable number of acid-fast elements, mainly short rods. This species grows fairly well in *p*- and *o*-cresol, and particularly so in *m*-cresol; in the last very short rods sometimes show acid-fast staining.

(c) *Development of aerial mycelium.* *P. paraffinae*, in the relative ease with which it forms aerial mycelium on a variety of media, approximates to the true actinomycetes (*Streptomyces*). Observation of microcultures on starvation media such as water agar, showed at 12 hr., simple vegetative mycelium; 20 hr., greater branching of mycelium, one aerial hypha; 42 hr., 5–10 aerial hyphae, all simple and undivided; 66 hr., aerial hyphae with irregular sub-division of contents, no distinction in staining properties from vegetative filaments. From month-old, dried-up plates of  $\text{MnSO}_4$  sucrose nitrate agar entirely covered with a veil of aerial mycelium, certain individual filaments were removed by means of a micromanipulator. They were all exceedingly fragile, and with the gentlest handling broke up into irregular cylindrical

elements which, when cultured in droplets of nutrient glucose broth, elongated to form a new mycelium indistinguishable from that produced by cells of the normal substratum growth.

#### DISCUSSION

Representatives of the other species listed at the beginning were also tested under similar conditions, and the picture was much the same. Acid-fast elements could be found in most strains on the same favourable media, and they could be intact mycelia, mycelial fragments, isolated branching filaments, short rods, or cocci, according to the nature of the species, the age and quality of its growth on the various substrates. No consistent type of acid-fast cell could be found for any species in the whole range, and no evidence for any peculiar forms of 'resting spores'. All the proactinomycetes were less resistant to acid ethanol decolorization than the true mycobacteria (*cf.* Umbreit, 1939). The gradual waxing and waning of the property of acid-fastness during prolonged cultivation in such media as milk suggests the validity of the theory of Yegian & Vanderlinde (1947) that this property is dependent upon the permeability of the cytoplasmic membrane. Micro-organisms which may grow out of the nutritive medium into the air, as do all the actinomycetes, frequently show at different stages of growth a patchy staining with vital dyes such as dilute methylene blue. Finally, the observations of previous workers that the aerial mycelium, when present, is not spore-bearing have been confirmed.

This work was done by the author as a member of the scientific staff of the Agricultural Research Council.

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## The Assimilation of Amino-acids by Bacteria

### 8. Trace Metals in Glutamic Acid Assimilation and their inactivation by 8-Hydroxyquinoline

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#### With a Note on Relative Dissociation constants of some Metal-oxine Complexes

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**SUMMARY:** The assimilation of glutamic acid by washed suspensions of *Staphylococcus aureus* is inhibited by 8-hydroxyquinoline (oxine). Fermentation and respiration are also inhibited but only at higher concentrations of oxine than those required for the complete inhibition of assimilation. The inhibition of assimilation by oxine can be annulled by washing the cells in water or in salts of certain divalent metals; manganese salts are more effective than those of Co, Fe or Mg. The inhibition produced by high concentrations of oxine can be decreased by metals known to form chelate complexes with oxine; the addition of an equivalent of Mn completely abolishes the inhibition.

Glutamate assimilation in *Staph. aureus* is impaired by a deficiency during growth of either Mn or Mg, but is not abolished unless both metals are absent. Washed suspensions of Mn- and Mg-deficient cells are activated by the addition of either Mn or Mg; Mg is more effective than Mn in activating washed cells whereas Mn is more effective than Mg in activating growing cells.

Assimilation of glutamic acid is activated by either Mn or Mg, Mn being utilized preferentially during growth. In the absence of Mn, the organisms utilize Mg and the sensitivity of assimilation to inhibition by oxine decreases.

Certain amino-acids are taken up by Gram-positive bacteria and are concentrated in the free state inside the cell before taking part in the cell metabolism (Gale, 1947*a*, 1947*b*; Gale & Mitchell, 1947; Taylor, 1947). Amino-acids may pass across the cell-wall into the cell by diffusion, as in the case of lysine, or by an active cell process as in the case of glutamic acid. It is this latter energy-requiring transfer of amino-acids which is inhibited by penicillin (Gale & Taylor, 1947) and the sensitivity of a bacterial cell to penicillin is determined by the dependence of its growth on these assimilatory processes, since cells which can synthesize their essential amino-acids, and are consequently independent of assimilation of the preformed amino-acids, are resistant to penicillin (Gale & Rodwell, 1948, 1949). Complete understanding of the action of penicillin and of the assimilation of amino-acids awaits the elucidation of the mode of active transfer across the cell-wall. Studies of the action of inhibitors of the transfer might help in the elucidation. It seems probable that any inhibitor whose primary action is on assimilation would have an antibacterial 'spectrum' similar to that of penicillin.

The present study was prompted by the finding of Albert, Rubbo, Goldacre & Balfour (1947) that 8-hydroxyquinoline (oxine) has such a 'spectrum'. These workers found that of the seven possible isomers of hydroxyquinoline, only 8-hydroxyquinoline was antibacterial and, further, was the only isomer able to form chelate complexes with metals. The inhibition of the growth of Gram-positive bacteria could be annulled by the addition to the medium of four equivalents of cobalt whereas that of Gram-negative bacteria was annulled by four equivalents of zinc, iron or, in some species, copper, but not by cobalt at that concentration. They suggested that inhibitors of this nature 'function by disorganising the trace-metal mechanism essential for bacterial growth and that this effect depends upon combination between the drug and some metals in the bacterial surface'.

Mellor & Maley (1947, 1948) studied the stability of co-ordination complexes formed by metals and various chelating agents and found that complexes with the divalent metals fell into the following order of decreasing stability: Pd, Cu, Ni, Co, Zn, Cd, Fe, Mn, Mg. The order appears to be approximately the same for all organic compounds forming such complexes. The authors do not quote values for oxine but Mitchell (see Note, p. 385) has confirmed that the metals Co, Fe, Mn, Mg fall into the same order of stability for the complexes with oxine.

#### MATERIAL AND METHODS

*The organism* was the strain *Staph. aureus* D used for the investigations on the inhibition of glutamic acid assimilation by penicillin (Gale & Taylor, 1947).

*General.* The growth media, preparation of bacterial suspensions and methods for assay of glutamic acid within the cells were as previously described (Gale, 1947 *a*). In general, *Staph. aureus* D was grown in a medium containing little free glutamic acid, the cells harvested after 6 hr. at 37° or 12 hr. at 30°, washed once and their internal free glutamic acid content measured; the cells were then suspended in buffered salt solution containing glutamic acid (200  $\mu$ l./ml.) and glucose (1.0 %) together with added test substances, and incubated at 37° in a final suspension strength corresponding to 1–2 mg. dry weight of organism/ml. After incubation for a given period, assimilation was stopped by rapid cooling to 0°, the cells centrifuged down, washed once in water and their internal glutamic acid content again measured. Concentrations of glutamic acid are expressed in  $\mu$ l. equivalent to CO<sub>2</sub> liberated; thus 22.4  $\mu$ l. = 1  $\mu$ mol.

*Metal-deprived medium.* This medium was made up as follows: Na<sub>2</sub>HPO<sub>4</sub>, 4.0 g.; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g.; NaCl, 1.0 g.; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 g.; arginine monohydrochloride, 1.0 g.; 10 % Marmite solution, 10.0 ml.; glass-distilled water, 980 ml. Glucose, 10.0 g. in 20 ml. water. The salts, arginine and Marmite were dissolved in 50 ml. glass-distilled water, and the pH adjusted to 8.5–9.0. The medium was then freed from polyvalent ions by alternate reaction with 8-hydroxyquinoline and extraction with chloroform by the method of Waring & Werkman (1942) except that about 50 mg. oxine were used for each treatment, the pH adjusted to an alkaline value and the process repeated five or six times. The first two or three chloroform extracts were green but the fifth was usually colourless. The efficiency of removal of ions such as Mn and Mg depends upon the

alkalinity of the medium and it is improbable that anything more than a partial removal of Mg is possible by this method. The glucose solution was similarly treated by oxine to remove metals; three treatments usually gave colourless chloroform extracts. The glucose was acidified with phosphoric acid and sterilized separately. The medium was made up to volume with glass-distilled water, the pH adjusted to 7.0 and solutions of trace elements added to replace those removed by the oxine treatment. These were used in the following final concentrations:  $\text{Mg}^{++}$ ,  $\text{m}/6 \times 10^3$ ;  $\text{Fe}^{++}$ ,  $\text{m}/6 \times 10^4$ ;  $\text{Mn}^{++}$ ,  $\text{m}/10^6$ ;  $\text{Co}^{++}$  and  $\text{Zn}^{++}$ ,  $\text{m}/6 \times 10^6$ . The metals other than manganese were used as sulphates ('Analar' grade, British Drug Houses, Ltd.); the magnesium sulphate was treated with oxine at pH 7.0 to decrease possible traces of manganese. Mg-free manganese sulphate was prepared from potassium permanganate. All glass-ware was freed from metals by the method of Waring & Werkman (1942) and the media were sterilized in Pyrex flasks capped by Pyrex glass beakers.

The media were aerated during growth by bubbling filtered air through glass capillary tubes. The inoculum was prepared from the cells of an 18 hr. culture in medium A (Gale, 1947*a*) which were centrifuged down, washed twice in glass-distilled water and suspended in metal-deprived medium. An inoculum of  $c. 10^7$  cells/l. medium was used and the cultures incubated at 30° for periods depending on the metal-deficiency of the medium (see below).

## RESULTS

### *Sensitivity of Staph. aureus D to oxine*

The threshold concentration of oxine inhibiting the growth of *Staph. aureus* D was determined by the method of serial dilution. It varied markedly with the nature of the medium. In medium A (casein digest, Marmite and glucose, Gale, 1947*a*) growth was inhibited for 48 hr. at 37° by 12–15  $\mu\text{g. oxine/ml.}$ ; in medium B (salts, Marmite and glucose) growth was inhibited by 1.0–1.5  $\mu\text{g./ml.}$  Since the two media differ mainly in their amino-acid concentration, the test was repeated in medium B to which vitamin-free acid-hydrolysate of casein (Ashe Laboratories) was added. With 0.1, 1.0 and 5.0 % casein hydrolysate, the threshold concentrations of oxine were 2.0, 4.0 and 10.0  $\mu\text{g. oxine/ml.}$  respectively.

### *Action of oxine on the accumulation of free glutamic acid within the cells*

When *Staph. aureus* is grown in a medium rich in glutamic acid, the concentration of free glutamic acid in the cells rises throughout growth and becomes steady when cell-division ceases (Gale, 1947*b*). The concentration within the cell at any moment is determined by the balance between the rate at which the amino-acid enters the cell and the rate at which it is metabolized within the cell. The rate of assimilation is approximately constant throughout the growth period whereas the rate of protein formation is greatest during the early stages of growth and ceases when growth ceases; the altering balance between the two processes gives rise to the increasing concentration of free glutamic acid within the cell as growth continues. The action of penicillin in

inhibiting assimilation was first indicated by the fact that the free glutamic acid concentration within the cells decreased rapidly in penicillin-treated cultures (Gale & Taylor, 1947). Fig. 1 shows the effect on the internal concentration of glutamic acid of adding a bacteriostatic concentration of oxine to *Staph. aureus* D growing in medium A. The increase in cell-mass and in internal glutamic acid concentration is normal for about an hour after addition of the

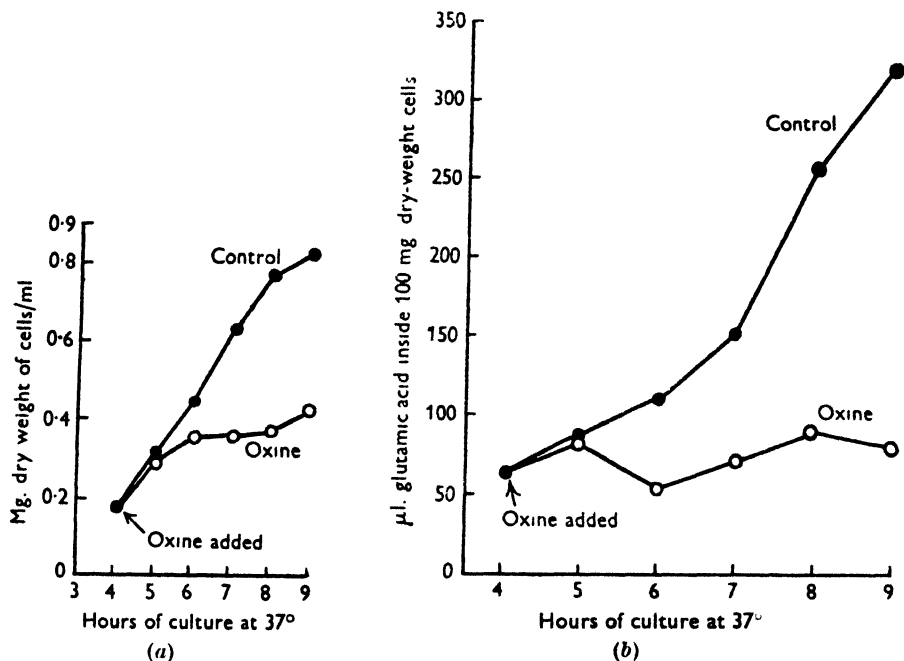


Fig. 1 (a) Effect of addition of oxine to medium on growth of *Staph. aureus* D. Growth medium A; 50  $\mu\text{g}$ . oxine/ml. added after 4 hr. growth at 37°. (b) Effect of addition of oxine to growing culture on the internal accumulation of glutamic acid. Growth medium A; 50  $\mu\text{g}$ . oxine/ml. added after 4 hr. growth at 37°; conditions as (a).

oxine; after this, growth is slowed and the internal concentration falls. The concentration of oxine (50  $\mu\text{g}$ ./ml.) was not sufficient to stop growth completely, because higher concentrations have complex actions on the general metabolism of the organism (see below). Four hours after the addition of oxine, growth starts again but the internal concentration of glutamic acid does not rise. This suggests that, as with penicillin, there has been interference with the passage of the amino-acid into the cell.

#### *Action of oxine on glutamic acid assimilation in washed suspensions of Staphylococcus aureus D*

The assimilation of glutamic acid can be measured in washed suspensions (Gale, 1947*a*). Penicillin does not affect assimilation by washed cells, it acts only on growing cells (Gale & Taylor, 1947). Oxine, on the other hand, inhibits glutamic acid assimilation in washed cells. Fig. 2 shows the increase in the internal concentration of free glutamic acid in the presence and absence of

oxine; concentrations of oxine which partially inhibit do so by diminishing the rate of passage of the amino-acid into the cell and not by decreasing the ability of the cell to accumulate glutamic acid. Fig. 3 shows the relation between the concentration of oxine and the inhibition of glutamate assimilation.

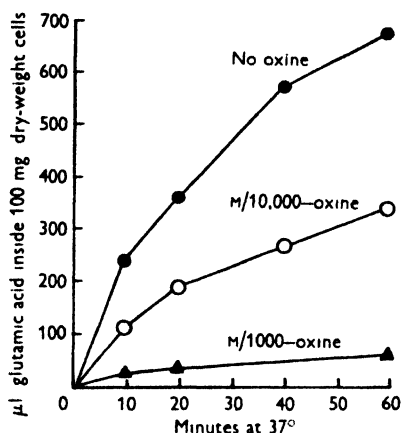


Fig. 2

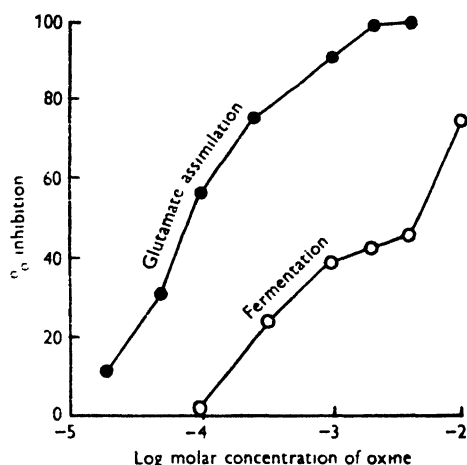


Fig. 3

Fig. 2. Effect of oxine on the course of glutamic acid assimilation by washed suspensions of *Staph. aureus* D. Organism grown in medium B for 16 hr. at 30°; cells made into washed suspension and incubated in buffered salt solution containing 1.0 % glucose and 200 µl. glutamic acid/ml. with oxine added as shown. Samples removed at intervals and assimilation stopped by rapid cooling to 0°.

Fig. 3. Inhibition of glutamic acid assimilation and of glucose fermentation in *Staph. aureus* D by addition of oxine to washed suspensions.

*Action of oxine on general metabolism.* The inhibition of assimilation by penicillin appears to be a specific effect as it occurs without affecting either the respiratory, fermentative or lysine-assimilation systems (Gale & Taylor, 1947). Table 1 shows the effect of 0.002 M oxine on certain metabolic activities of the

Table 1. *Metabolic activities of normal Staphylococcus aureus cells in the presence and absence of 0.02 M 8-hydroxyquinoline*

|  | Normal cells alone | Cells in presence of 0.002 M oxine |
|--|--------------------|------------------------------------|
| Respiration, $Q_{O_2}$                                 | 8                  | 6                                  |
| Glucose oxidation, $Q_{O_2}$                           | 75                 | 38                                 |
| Glucose fermentation, $Q_{CO_2}^{acid}$                | 93                 | 63                                 |
| Lysine assimilation (µl./100 mg. dry wt. cells)        | 44                 | 36                                 |
| Glutamic acid assimilation (µl./100 mg. dry wt. cells) | 440                | 30                                 |

organism, tested as described by Gale & Taylor (1947); at this concentration there is complete inhibition of glutamic acid assimilation and approximately 40 % inhibition of respiration and fermentation. Fig. 3 shows the relation



between oxine concentration and inhibition of fermentation; assimilation is markedly more sensitive than the glycolytic system; 0.0001 M oxine produces 60 % inhibition of the former while having no action on the latter, and the oxine concentration producing 50 % inhibition is 0.00008 M for assimilation and 0.0045 M for fermentation.

*Reversal of oxine inhibition.* The growth curves in Fig. 1 indicate that cells recover from oxine inhibition. To test whether the inhibition of assimilation is reversible, washed cells were incubated in 0.002 M oxine for 30 min. at 37°, centrifuged, and suspended in the usual buffer + glutamate + glucose medium. In control suspensions containing oxine there was complete inhibition of assimilation and 61 % inhibition of fermentation. With cells removed from the oxine solution, the inhibition of assimilation fell to 15 % and the inhibition of fermentation was completely abolished. Thus the inhibition can be reversed by removal of the oxine and resuspension of the cells in the usual salt mixture.

*Reversal of oxine inhibition by metals.* Albert *et al.* (1947) reversed growth-inhibition by oxine by adding to the medium four equivalents of cobalt. Since cobalt forms stable chelate complexes with oxine (Albert & Gledhill, 1947) this reversal might be due either to inactivation of the oxine by the cobalt or to replacement by cobalt of an essential metal inactivated or removed by oxine. If the former explanation were correct, then other complex-forming metals should be effective. This possibility was tested as follows: it was shown above that when cells were incubated in 0.002 M oxine for 30 min. at 37°, the inhibition was completely abolished by centrifuging the cells out of the oxine solution and resuspending them in buffered salt solution. When the preliminary incubation is carried out for 60 min. at 37° in 0.004 M oxine and the cells then washed once in water, neither assimilation nor fermentation recover completely but take place at rates *c.* 30–40 and 80 % respectively of the rates in untreated cells. The effect on assimilation and fermentation of washing these partially inhibited cells in solutions of various metallic sulphates can therefore be tested. In previous experiments, assimilation was tested in a buffered salt medium containing  $\text{MgSO}_4$  (Gale, 1947*a*), but since magnesium forms weak complexes with oxine, it has been omitted from further experiments and the tests made in a medium containing 0.1 %  $\text{KH}_2\text{PO}_4$ , 0.33 %  $\text{Na}_2\text{HPO}_4$  and 0.1 % NaCl.

Table 2 shows the effect of washing the partially inhibited cells with solutions containing metals known to form complexes with oxine and to be of biological interest. The residual inhibition of assimilation is completely annulled by cobalt, ferrous iron and manganese and 70 % reversal is obtained with magnesium. The metals fall into the following order of decreasing efficiency: Mn, Co, Fe, Mg. The comparable results in column (c), Table 2, show that Mn is markedly more effective than Co at equal concentration (0.0004 M). This order resembles the order of stability of chelate complexes as determined by Mellor & Maley (1947, 1948; see above) except for the position of Mn, Cu and Zn. However, in parallel fermentation tests (Table 2) whereas Co, Mn, ferrous iron, and Mg all completely annulled the residual inhibition of fermentation, Cu and Zn in the concentrations tested abolished all fermentation. Attempts were made to use lower concentrations of these two metals but the glucose fermentation was inhibited

90 % by 0.00001 M-CuSO<sub>4</sub> or by 0.0002 M-ZnSO<sub>4</sub> so that concentrations of the order necessary to antagonize the oxine completely inhibit the fermentation. Since, under the conditions of test, fermentation provides the energy for the

Table 2. *Removal of oxine inhibition by washing with solutions of metallic ions*

*Staph. aureus* D was grown in medium B for 6 hr. at 37° and the cells made into washed suspension. The cells were incubated for 1 hr. at 37°, at a final suspension strength of 1-2 mg. dry weight/ml., in phosphate buffer pH 7.0 containing 0.004 M oxine and then centrifuged down. Samples of the partially inhibited cells were then washed once, either in distilled water or in metal solutions as below. The ability of the cells to assimilate glutamic acid was then tested in each case; the ability to ferment glucose was also tested by following the gas production in bicarbonate/CO<sub>2</sub> buffer system. If the glutamate assimilation of the untreated cells = X, that of the oxine-treated cells washed in water = Y, and that of the oxine-treated and metal-washed cells = Z, the percentage reversal of oxine inhibition as expressed below = 100 (Z - Y)/(X - Y). Comparable results are given in the same vertical column.

| Metal             | Concentration<br>during washing<br>(M) | Percentage removal of oxine<br>inhibition |     |     | Inhibition of<br>fermentation as<br>percentage rate<br>in control |
|-------------------|--|---|-----|-----|---|
|                   |  | (a)                                       | (b) | (c) |   |
| Copper            | 0.001                                  | 0   | —   | —   | 100   |
|                   | 0.0002                                 | 0   | —   | —   | 100   |
| Cobalt            | 0.01                                   | 57  | —   | —   | 28  |
|                   | 0.001                                  | 86  | 60  | —   | 0   |
|                   | 0.0004                                 | —   | 100 | 52  | 0   |
|                   | 0.0001                                 | 10  | —   | —   | 0   |
| Zinc              | 0.001                                  | 0   | —   | —   | 100   |
| Fe <sup>++</sup>  | 0.001                                  | 94  | 54  | 40  | 0   |
|                   | 0.0004                                 | —   | 42  | —   | 0   |
| Fe <sup>+++</sup> | 0.001                                  | 40  | —   | —   | 0   |
| Manganese         | 0.0004                                 | —   | —   | 98  | 0   |
| Magnesium         | 0.001                                  | 34  | —   | —   | 0   |
|                   | 0.004                                  | 51  | —   | 67  | 0   |
|                   | 0.01                                   | —   | 42  | —   | 0   |

transfer of glutamic acid into the cells, there can be no recovery of assimilation in these cases. The inhibitory concentrations of Cu and Zn were not significantly lowered by the presence of oxine, so it appears that the fermentation enzymes in the cell have a higher affinity than oxine for these metals.

With the exception of Mn, the efficacy of the various metals in annulling oxine inhibition can be correlated with the stability of the chelate complexes; Mg forms the least stable complex and is the least effective. The data suggest that oxine combines reversibly with some substance in the bacterial cell and so inhibits glutamic acid assimilation; the presence of polyvalent metals in the environment results in withdrawal of the oxine from the complex within the cell, the efficiency of this action being determined by the relative stability of the chelate complexes inside and outside the cell.

*Decrease of oxine inhibition by metals.* The nature of the bacterial factor that reacts with oxine might be indicated by investigating the effect on assimilation of high concentrations of oxine in the presence of various metals with which it forms complexes. 0.004 M oxine was used and an equivalent concentration of various metals added. In most cases this produced heavy precipitates of

chelate compounds, but the precipitate did not interfere with the tests. Oxine alone completely inhibited assimilation, but the inhibition was diminished by the simultaneous presence of metals such as Co, Fe, Mn or Mg. The diminution was greatest when the metals were present in concentrations equivalent to the oxine. In some cases higher concentrations of metals gave inhibitions greater than that due to oxine alone, but these effects again corresponded to inhibition of the fermentation system. In concentrations equivalent to oxine, the metals

Table 3. *Diminution of oxine-inhibition of glutamic acid assimilation by the presence of metals*

*Staph. aureus* D was grown for 6 hr. at 37° in deficient medium B and the cells made into washed suspension. The ability to assimilate glutamic acid was then tested by incubating for 1 hr. in buffered salt solution (heavy-metal-free), glucose (1.0 %) and glutamic acid (200  $\mu$ l./ml.) and measuring the internal glutamic acid concentration before and after incubation. In parallel experiments, the assimilation was measured in the presence of 0.004 M oxine alone and in the presence of various metals, as their sulphates, as below. Results are expressed as percentage inhibition of assimilation, 0.004 M oxine causing 100 % inhibition. In each case, parallel determinations were made of the glucose fermentation of the organism.

| Metal             | Concentration<br>(M) | Inhibition of<br>glutamic acid<br>assimilation<br>(%) | Inhibition of<br>glucose<br>fermentation<br>(%) |
|-------------------|----------------------|---|---|
| (Oxine alone)     | 0.004                | 100   | 40  |
| Cobalt            | 0.001                | 83  | —   |
|                   | 0.002                | 25  | 32  |
|                   | 0.004                | 55  | —   |
|                   | 0.02                 | 100   | 100   |
| Fe <sup>++</sup>  | 0.001                | 76  | —   |
|                   | 0.002                | 48  | 15  |
|                   | 0.004                | 94  | 92  |
|                   | 0.002                | 79  | 75  |
| Fe <sup>+++</sup> | 0.002                | 0   | 17  |
| Manganese         | 0.002                | 87  | 20  |

Values in italics represent those obtained when metallic-ion concentration was equivalent to oxine concentration.

decreased the inhibition in proportion to the stability of the corresponding oxine-metal complexes. Thus Co diminished the oxine inhibition by 75 % whereas Mg diminished it by c. 10 %, and iron was intermediate with diminution by 52 % (Table 3). However, Mn was again the most effective metal as, at a concentration equivalent to the oxine, it completely annulled inhibition of assimilation. Mn is thus better than Co and its activity cannot be correlated with its position in the stability series.

If the oxine is distributed between the bacterial factor and the metal in the environment in these experimental systems, the distribution is presumably determined by: (1) the relative affinities of the factor and of the metal for oxine; (2) the relative concentrations of the factor and the metal. Since the inhibition can be partly or completely removed by Mg at concentrations approximately the same as those effective in the case of Co, it is probable that the stability of the bacterial factor-oxine complex cannot be markedly greater than that of the magnesium-oxine complex. It is also probable that, when the oxine inhibition

is decreased by the presence of metal, if the metal added to the environment were identical with the bacterial factor, then inhibition would be completely abolished, since there would always be an excess of functional metal over inhibitor. This appears to be the case for Mn, which is the most effective metal in reversing inhibition (Table 2) and completely abolishes inhibition when present in amounts equivalent to the oxine (Table 3). It is interesting that, although Mn completely abolishes oxine inhibition of assimilation, it is less effective in decreasing the inhibition of fermentation.

#### *Metals involved in glutamic acid assimilation*

*Effect of metal-deprivation of growth medium.* The results described in the first section of this communication suggest that a metal is involved in the assimilation of glutamic acid by *Staph. aureus* and that this metal can combine reversibly with oxine. The identity of the metal may be established by removing metals one at a time from the medium and observing the effect on assimilation. Since the hypothetical metal combines with oxine, the method of Waring & Werkman (1942) for the removal of polyvalent metals from bacterial media is particularly suitable. The method was used by these authors for simple chemically defined media and is not so effective for complex media such as medium B used in these studies. However, when modified as described above, the method was sufficiently successful with medium B to give the following significant effects.

A large batch of medium was thoroughly 'stripped' of polyvalent metals, and divided into seven batches of 600 ml.;  $\text{Cu}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Mn}^{++}$  and  $\text{Mg}^{++}$  in the concentrations given on p. 371 were added, one different metal being omitted from each of six batches, the seventh batch containing all six metals. The batches were then inoculated and incubated; growth took place in all batches after a lag of 10–16 hr. The cells in each case were harvested when the turbidity was equal to that of a 12 hr. culture in medium B under normal conditions. The cells were washed once in glass-distilled water and their ability to assimilate glutamic acid tested by incubation for 45 min. in buffer solution (free from heavy metals) containing 1.0 % glucose and glutamic acid (200  $\mu\text{l.}/\text{ml.}$ ). The increase in the internal concentration of glutamic acid during incubation was not affected by the omission from the medium of Co, Cu, Zn; the increase in concentration was smaller in the Fe-deficient cells than in the control with all metals, and the increase was insignificant in the Mn- or Mg-deficient cells. The initial internal concentration before incubation with glutamic acid was higher in the Fe-deficient cells than in the other cells and it seemed probable that the smaller increase in the former on incubation was due to the fact that the initial concentration already approached saturation. To test this point, the rate of assimilation was determined in cells grown in media deficient in Fe, Mn, Mg, and no metals, respectively. Fig. 4 shows the results; the rate of glutamate assimilation is practically the same in the Fe-deficient as in the control cells, whereas it is markedly decreased in cells harvested from either Mn- or Mg-deficient media. The fermentation activity of the cells from the four cultures was determined; the rate was not significantly affected by either Fe- or Mn-deficiency whereas the rate in Mg-deficient cells was low.

*Effect of manganese and magnesium deficiency on assimilation and fermentation*

The results in Fig. 4 indicate that deficiency of either Mn or Mg impairs assimilation. The removal of Mg from the medium with oxine is unlikely to be highly efficient, since Mg forms chelate complexes of low stability. However,

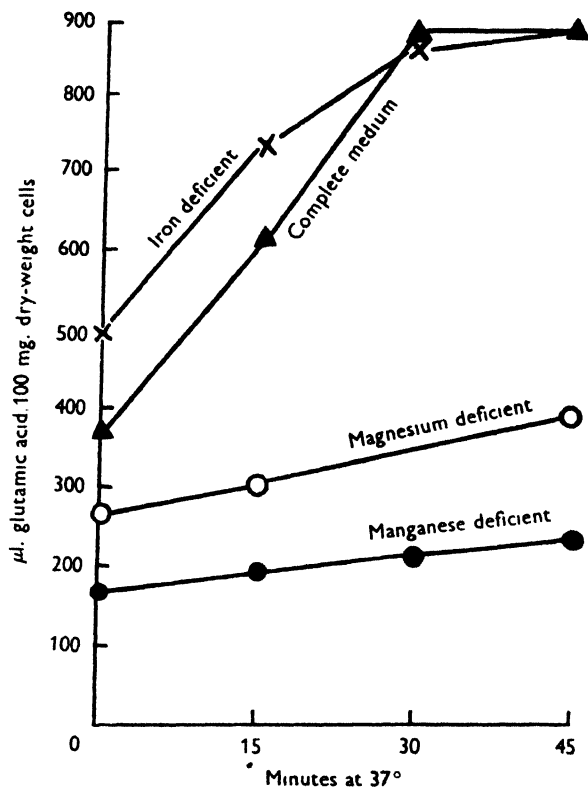


Fig. 4. Rate of glutamic acid assimilation in metal-deprived cells. Organism grown in medium B deprived of metals by oxine treatment; the metals were then replaced with exceptions as below and the rate of glutamic acid assimilation measured for washed suspensions of cells harvested from the various cultures. Fermentation rates were determined on each suspension by measurement in Warburg manometers of the  $\text{CO}_2$  evolution in the presence of glucose and  $\text{CO}_2$ /bicarbonate buffer.  $\blacktriangle$ — $\blacktriangle$  All metals replaced,  $Q_{\text{CO}_2}^{\text{acid}}$  (glucose)=85.  $\times$ — $\times$  Fe-deficient culture,  $Q_{\text{CO}_2}^{\text{acid}}$ =87.  $\circ$ — $\circ$  Mg-deficient culture,  $Q_{\text{CO}_2}^{\text{acid}}$ =26.  $\bullet$ — $\bullet$  Mn-deficient culture,  $Q_{\text{CO}_2}^{\text{acid}}$ =72.

the normal Mg requirements are high and by treating the medium with oxine at *c.* pH 9 it proved possible to lower the Mg concentration sufficiently to obtain significant effects, although these varied widely from batch to batch as judged by the length of the lag phase of growth and by the rate of fermentation of the harvested cells. In media deprived of both Mn and Mg, the lag phase varied from 5–20 hr. and the rate of fermentation was sometimes lowered to 30 % of that in the Mg-rich control. In two of 28 tests, no growth took place in the deprived medium, though it took place in portions of the same medium to which either Mn or Mg had been added. The Mn-tolerance of the organism

appeared to vary with the ionic balance in the medium because, although 40  $\mu\text{g}$ . Mn/ml. was tolerated in the full medium, concentrations of 0.5–1.0  $\mu\text{g}$ ./ml. markedly decreased the rate of growth in Mg-deficient media.

Table 4. *Effect of Mn and Mg on rate of glutamic acid assimilation in deficient Staphylococcus aureus*

*Staph. aureus* D grown in medium B previously treated with oxine to remove metals and Mn or Mg then replaced as below. The deficient cells were made into washed suspension and the internal glutamic acid concentration determined. Samples were then incubated for 20 min. at 37° in buffered salt solution containing 1 % glucose, 200  $\mu\text{l}$ . glutamate/ml. and Mg or Mn as below. After 20 min. the samples were rapidly iced, centrifuged down and the new internal concentration assayed. The rate of assimilation is expressed as  $\mu\text{l}$ . glutamic acid increase in internal concentration/100 mg. cells/20 min. Parallel determinations were made upon the fermentation rates of the cells.

| Series | to growth medium   |         | Rate of glutamate assimilation in presence of |            |             | Fermentation of glucose<br>$\text{QCO}_2$ |
|--------|--------------------|---------|---|------------|-------------|---|
|        | Mn (M)             | Mg (M)  | No addition                                   | 0.001 M Mn | 0.0001 M Mg |   |
| 1      | 0.00005            | 0.00017 | 224   | —          | —           | —   |
|        | 0.0000042          | 0.00017 | 106   | —          | —           | 70  |
|        | 0.000001           | 0.00017 | 156   | 220        | 200         | 66  |
|        | 0.000001           | 0.00017 | 78  | 164        | —           | 82  |
|        | Nil                | 0.00017 | 114   | —          | —           | —   |
|        | Nil                | 0.00017 | 88  | 152        | 88          | 72  |
| 2      | Nil                | 0.0001  | 20  | 108        | 74          | 66  |
|        | Nil                | 0.0001  | 21  | 52         | —           | 60  |
|        | Nil                | 0.0001  | 14  | 93         | 93          | —   |
|        | Nil                | 0.0001  | 0   | 30         | 0           | 56  |
| 3      | 0.000001           | Nil     | 129   | 235        | 173         | 70  |
|        | 0.000001           | Nil     | 112   | 240        | 240         | 46  |
| 4      | Nil                | Nil     | 8   | 143        | 73          | 36  |
|        | Nil                | Nil     | 0   | 150        | 150         | 48  |
| 5      | Untreated medium B |         | 280   | 302        | 240         | 106                                       |

Cells were grown in metal-free media to which various concentrations of Mn and Mg had been added. They were harvested early in the growth period and the rate of glutamic acid assimilation, and in most cases the rate of glucose fermentation, were determined; the results are shown in Table 4. The experiments were divided into four series: (i) the concentration of Mg was kept constant at M/6000 while that of Mn was decreased; (ii) Mg only was added, in sub-optimal concentrations; (iii) Mn was added but not Mg; (iv) neither Mn nor Mg was added. In the first series, the rate of assimilation decreased as the Mn content of the medium decreased but there was a significant activity even when no Mn was added. Assimilation was, however, further decreased and sometimes abolished when the Mg content was also decreased, as in the second series. The addition of Mn to M/10<sup>6</sup> to a medium without Mg gave an assimilation rate approximately half that attained in the normal untreated medium (series 3 and 5, Table 4), whereas there was little or no assimilation in cells harvested from media deficient in both Mn and Mg. It seems from these results that either Mn, or Mg, or both, are involved in the glutamic acid assimilation. However, the energy necessary for the transfer of glutamic acid into the cells

is derived from glycolysis, and Mg is known to be involved in several of the stages of the glycolytic cycle. It can be seen that Mg-deficiency is accompanied by a fall in the fermentation rate, and that assimilation is not completely stopped by Mn-deprivation alone. This suggests that Mg is involved in assimilation apart from its role in fermentation.

**Reactivation of Mn- and Mg-deficient cells by metals.** Tests were carried out on cells grown in media deprived of both Mn and Mg to determine whether assimilation was reactivated by metals added to the washed suspension. Co,

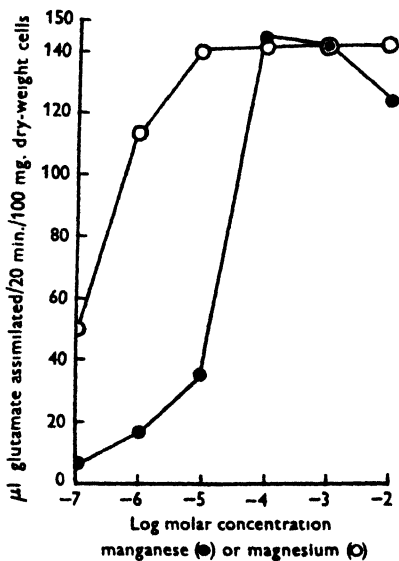


Fig. 5

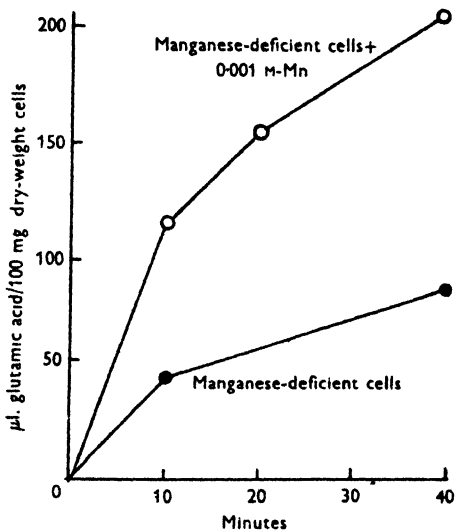


Fig. 6

Fig. 5. Activation of washed suspensions of cells deficient in Mn and Mg by Mn and Mg. *Staph. aureus* D grown in medium B deficient in Mn and Mg; cells harvested and made into washed suspension and the rate of glutamic acid assimilation over 20 min. at 37° determined in the presence of either Mn or Mg as shown.

●—● Mn. ○—○ Mg.

Fig. 6. Activation of glutamic acid assimilation in Mn-deficient cells by Mn. *Staph. aureus* D grown in Mn-deficient medium B; cells harvested and made into washed suspension, incubated in buffered salt solution containing 1.0 % glucose, 200 μl. glutamic acid/ml. with and without 0.001 M-MnSO<sub>4</sub>, samples taken at intervals as shown, cooled rapidly to 0° and internal glutamate concentration measured.

Fe and Zn had no effect on assimilation but 0.001 M-MgSO<sub>4</sub> or MnSO<sub>4</sub> markedly increased it (Fig. 5). Mg was more effective than Mn although both metals produced the same effect in optimal concentrations; Mn and Mg together in optimal concentrations had no greater effect than either alone. Table 4 shows the rates of assimilation obtained in washed deficient cells by the addition of m/1000 Mn or m/10<sup>4</sup> Mg. In all cases Mn or Mg increased the rate, often to that of normal cells. In some cases Mg was less effective than Mn, but since the tests were made with the one concentration of metal only and since the sensitivity of the deficient cells to these metals is not constant from one batch

to another, it is probable that the smaller effect of Mg was due in some cases to its presence in suboptimal concentrations.

These results suggest that either Mn or Mg is involved in assimilation but that Mg is more effective than Mn in activating washed suspensions. The analysis of the inhibition of assimilation by oxine suggests strongly that in the normal cell Mn either is the metal concerned or can replace it. Further, since assimilation is a thousand times more sensitive than fermentation to oxine, and since oxine will not chelate to any extent with Mg at the experimental pH, the evidence points to Mn as the metal concerned in the normal cell. Mn certainly activates assimilation in deficient cells (Fig. 6) but Fig. 5 indicates that if both

Table 5. *Effect of washing on rates of assimilation*

*Staph. aureus* D was grown in medium B of controlled Mn or Mg content. In experiments A, the cells were harvested and their rate of glutamate assimilation ( $\mu$ l. glutamic acid/100 mg. dry wt./20 min.) determined on a portion of the cells before any washing; a further portion was then washed twice in glass-distilled water at a final suspension strength of approximately 0.3 mg. dry wt. cells/ml. and the assimilation rate then determined. In experiments B, the cells were grown in media deficient in both Mn and Mg; after harvesting the cells were incubated for 15 min. at 37° in buffered salt solution containing 0.25% glucose and either 0.001 M-Mn or 0.0001 M-Mg; they were then centrifuged down and the assimilation rate determined on half of the cells without washing, the other half was then washed twice in glass-distilled water as above and the rate then determined.

| Experiment | Metal content of growth medium |           | Pretreatment with metal | Rate of assimilation ( $\mu$ l. glutamate/100 mg. dry wt. cells/20 min.) |               |
|------------|--------------------------------|-----------|-------------------------|--|---------------|
|            | Mn                             | Mg        |                         | Before washing   | After washing |
| A          | 0.000001 M                     | Nil       | None                    | 139  | 147           |
|            | Nil                            | 0.00017 M | None                    | 129  | 104           |
| B          | Nil                            | Nil       | 0.001 M-Mn              | 206  | 206           |
|            | Nil                            | Nil       | 0.0001 M-Mg             | 206  | 148           |

Mg and Mn were present, Mg would be the more effective. Moreover Mn- and Mg-deficient cells that cannot assimilate are activated as well by Mg as by Mn (Table 4). It is possible that Mg diffuses in and out of the cell more readily than Mn and is more easily washed out of the cell. This was tested, firstly, by determining the effect of exhaustive washing on the assimilation rates of cells grown in Mn-without-Mg and in Mg-without-Mn. Secondly, cells were grown in media deficient in both Mn and Mg, incubated with optimal concentrations of either Mn or Mg, washed exhaustively in water, and their assimilation rates measured (Table 5). Washing had no effect on the cells grown in, or activated by, Mn and very little effect on those grown in, or activated by, Mg.

It is possible that the permeability of washed cells to metals may differ from that of growing cells, in which case the greater sensitivity to Mg shown in Fig. 5 might be due to the easier entrance of the Mg ion into the washed cell as compared with the larger Mn ion. The data in Table 4 suggest that Mn in the growth medium is effective at much lower concentrations than those needed to activate washed suspensions. To test this, organisms grown in a medium deficient in both Mn and Mg were incubated for 1 hr. at 37° in a modified



growth medium containing either Mn or Mg. The medium was composed of buffered salt solution with aneurin, nicotinamide, 0.1 % vitamin-free casein-hydrolysate lacking glutamic acid, and 0.5 % glucose, the whole being deprived of heavy metals by oxine treatment. To keep the internal concentration of glutamic acid low at this stage, the casein hydrolysate used was treated with glutamic decarboxylase until it was free from glutamic acid.

After the 1 hr. period of incubation, during which some growth, if not cell-division, might be expected to occur, the cells were centrifuged down and resuspended for the usual assimilation test. Under these conditions, the deficient cells again were activated by either Mn or Mg, but Mn was now much more effective than Mg (Fig. 7). The activation-concentration curve for Mg is approximately the same in washed or growing cells whereas Mn is c. 2500 times more effective in growing cells. It follows that if the cells grow in the presence of both Mn and Mg, the assimilation mechanism will be preferentially activated by Mn.

#### *Oxine sensitivity of cells grown in the presence of Mn or Mg*

The results so far obtained indicate that the assimilation of glutamic acid involves either Mn or Mg and that, although Mn is utilized preferentially during growth, its place can be taken by Mg. At pH 7 the stability of the Mg-oxine complex is much less than that of the Mn-oxine complex, so that it might be expected that the assimilation by cells grown in media containing Mg but deprived of Mn would be less sensitive to oxine than that of cells grown in media containing Mn but deficient in Mg. Fig. 8 shows the effect of oxine concentration on the rate of assimilation by cells grown (*a*) in normal untreated medium, (*b*) in medium deprived of Mg but supplied with Mn, and (*c*) in medium deprived of Mn but supplied with Mg. The sensitivity of the cells of culture (*c*) is significantly less than that of cells from either (*a*) or (*b*) which have substantially the same sensitivity. As Mg replaces Mn in the culture, the sensitivity to oxine of assimilation approaches that of fermentation, in which the principle metal is Mg.

#### DISCUSSION

The studies of Albert *et al.* (1947) on the antibacterial action of 8-hydroxy-quinoline and related compounds strongly support the suggestion (Albert, 1944) that these substances act by combining with trace metals which are essential in bacterial metabolism and thereby prevent these metals performing their metabolic functions. The inhibition of glutamic acid assimilation in *Staph. aureus* by oxine and the partial or complete removal of this inhibition by certain metallic ions are consistent with the hypothesis that some metal which can combine with oxine forms an essential part of the assimilation mechanism and that this metal is inactivated by chelation with oxine. Two methods of inactivation seem possible: either the oxine combines with and inactivates the metal *in situ*, or the oxine combines with the metal and removes it from the organism. Since the metal-oxine complexes are more soluble in lipids than in water, it seems improbable that the complex would pass easily out of the organism into the medium. Further, the complete restoration of assimilation

by washing the oxine from the cells suggests that the oxine combines with the metal within the organism but, when washed away, leaves the metal behind. From an analysis of the effects of metals in annulling oxine inhibition of assimilation, it has been shown above that the metal concerned in the normal cell is probably manganese.

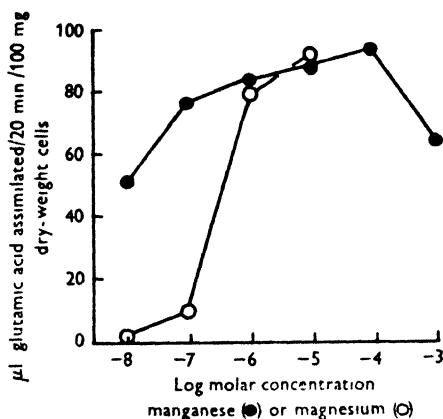


Fig. 7

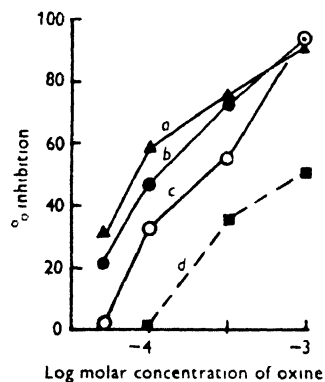


Fig. 8

Fig. 7. Activation of glutamic acid assimilation in cells deficient in Mn and Mg by incubation in a modified growth medium containing Mn or Mg. *Staph. aureus* D grown in medium B deprived of both Mn and Mg; cells harvested and incubated for 1 hr. at 37° in the following medium with addition of either Mn or Mg as above: buffered salt solution; 0.1% casein hydrolysate treated to remove vitamins, metals and glutamic acid; 0.5% glucose; aneurin 1 μg./ml.; nicotinamide 1 μg./ml. After incubation the cells were centrifuged down and resuspended in buffered salt solution containing 1.0% glucose and 200 μl. glutamic acid/ml. for 20 min. at 37°. At the end of this period, the cells were harvested, washed and the increase in internal glutamic acid concentration determined.

Fig. 8. Inhibition of glutamic acid assimilation by oxine; dependence on nature of metals present during growth. *Staph. aureus* D grown in: (a) medium B; (b) medium B deprived of Mg but containing  $v/10^6$ -Mn; (c) medium B deprived of Mn but with  $m/6000$ -Mg added. Assimilation inhibitions determined with washed suspensions in each case. Curve (d) shows oxine inhibition of glucose fermentation for cells of culture (c).

The results obtained with metal-deficient cultures indicate that either Mn or Mg is involved in assimilation. Mg is known to be necessary for the glucose fermentation system which provides energy for the transfer of glutamic acid across the cell-wall, but the results indicate that it can also play a part in the assimilation mechanism itself. Mg is known to activate many enzyme systems and is usually replaceable by Mn, although Pollock & Wainwright (1948) have recently obtained a growth effect on a Gram-negative organism which appears to be Mg-specific. When tests indicate that a system may be activated by either Mn or Mg, it is difficult to decide which metal is the natural activator *in vivo*. If the metals act in an ionic form then they are presumably freely interchangeable whether *in vivo* or *in vitro*, but if co-ordination complexes are involved within the organism, then the nature of the metal used 'naturally'

will depend upon the availability of the metal and the relative stabilities of the complexes formed. Mellor & Maley (1948) found that the order of stability of metal co-ordination complexes is approximately the same whatever the nature of the organic part of the complex; consequently if the formation of such complexes is involved, Mn will compete successfully with Mg and will be taken up preferentially. Both Mn and Mg can activate assimilation in washed cells, although Mg is more effective at low concentrations. This does not correlate with the argument developed above that the metal chelated with oxine in 'normal' cells is Mn, nor is it consistent with the marked difference in the oxine sensitivity of assimilation and fermentation in 'normal' cells. However, if activation of assimilation is carried out in a medium in which growth can occur, then Mn is 2500 times more effective than in a simple salt solution so that, in the growth medium, Mn is 30–50 times more effective than Mg. It seems probable that harvested and washed cells are less permeable to Mn than are cells growing and dividing.

It seems probable that the assimilation mechanism involves a metal whose function can be filled by either Mn or Mg and that, when the cell grows in a medium containing both Mn and Mg, Mn is preferentially co-ordinated. Mn forms a chelate complex with oxine which acts as a reversible inhibitor of assimilation. When growth takes place in a medium deficient only in Mn, then the mechanism becomes activated by Mg, but this results in a decrease in its sensitivity to oxine (see Fig. 8).

The present work was prompted by the finding of Albert *et al.* (1947) that oxine has an antibacterial 'spectrum' similar to that of penicillin and it has now been shown that both penicillin and oxine inhibit the transfer of glutamic acid into *Staph. aureus*. The mechanism of inhibition is almost certainly not the same for the two substances. This follows since (i) penicillin has no action on assimilation in washed cells where oxine causes complete inhibition, (ii) the inhibition of assimilation by growth in the presence of penicillin is irreversible, whereas the oxine inhibition is easily removed, (iii) oxine also has complex inhibitory actions on respiration and fermentation whereas penicillin has not at concentrations at least a thousand times greater than that required to inhibit assimilation, (iv) strains of *Staph. aureus* rendered resistant to penicillin are as sensitive to oxine as penicillin-sensitive strains, due perhaps to the non-specific nature of oxine as an inhibitor.

## A Note on the Relative Dissociation Constants of some Metal-oxine Complexes

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The relative dissociation constants of the Mg-, Mn-, and Co-oxine complexes cannot easily be determined in aqueous solution because of their very low solubilities. When 10 ml. of M/1000 oxine in amyl alcohol are equilibrated against 10 ml. of aqueous M/10,000 metal chloride, the concentration of metal complex in the amyl alcohol, and thus its degree of dissociation, may be determined colorimetrically. Because hydrogen ions compete with the metal ions for the oxine, the dissociation of the complex is a function of the pH value of the aqueous phase. Under these conditions, the pH value at which the complexes are half dissociated was found to be pH 5 for cobalt complex and pH 7 for the manganese complex. Taking into account the divalency of the metal, the apparent dissociation constants must, therefore, differ by a factor of about  $10^4$ , the manganese being more dissociated than the cobalt complex.

The relative value for the magnesium complex could not be determined in this way, because it forms nearly colourless solutions and this only at very alkaline pH values. Because of the pale colour of the solutions of the complex, however, it was possible to use direct competition against manganese. It was found that at pH 10, the manganese complex formed by equilibrating 10 ml. of M/10,000 aqueous manganous chloride against 10 ml. of M/1000 oxine in amyl alcohol was half dissociated by a concentration of M/100 magnesium chloride in the aqueous phase. Thus, the apparent dissociation constants differ by a factor of about 100, the magnesium complex being more dissociated than the manganese complex. The values obtained for the relative apparent dissociation constants in this way do not, of course, represent simple dissociation constants because they involve also the amyl alcohol-water partition coefficient for the oxine and metal complexes. But this should not be any disadvantage from the point of view of the biological material considered in this paper.

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## The Relationship of Certain Branched Bacterial Genera

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**SUMMARY:** The aerobic sporing *Streptomyces* may be distinguished from the parasitic anaerobic *Actinomyces* by the type of branching which they possess. It is proposed that they should be assigned to separate orders, Streptomycetales and Actinomycetales. The latter order is subdivided according to the type and arrangement of the component cells, and consists of two Families. The first, Actinomycetales, includes anaerobic actinomyces, and also 'soil diphtheroids' for which a new genus, *Jensenia* is proposed. The second, Mycobacteriaceae, includes *Mycobacterium*, *Corynebacterium* and *Nocardia*.

The true relationship of those bacterial genera which have, at various times, been grouped with the *Actinomyces*, is not easy to establish. In most early classifications the character of branching, however occasional, has been given great weight, although the techniques employed for the definition of this character have not always been adequate to the task; many genera, having little else in common, have been classed together for this reason. More recent amendments of the original schemes have been based largely upon the original, inadequate evidence (Waksman & Henrici, 1943). A return to the primary basis of classification, morphology, and a re-examination of the evidence in this respect, might remove some of the anomalies of the present position.

The name *Actinomyces* was originally given to the filamentous organism which was responsible for actinomycosis in animals; later the fungus-like organisms, now often called *Streptomyces*, were included in the genus. The present classification of Bergey (1948) restores the distinction between the two groups but retains them in a single order.

Lehmann & Neumann (1896) proposed the genera *Corynebacterium* and *Mycobacterium*, primarily for the pathogenic bacteria causing diphtheria, tuberculosis and leprosy. Later the genus *Corynebacterium* was extended to include almost any Gram-positive, non-motile, non-sporing bacterium showing any morphological irregularity; and the genus *Mycobacterium* to include any acid-fast organism.

As indicated by Conn & Dimmick (1947), each genus had species assigned to it which differed more from other species in the same genus than did the type species from one another. The classification of Bergey (1948), which limits the genus *Mycobacterium* to *M. phlei*, *M. lacticola* and the usual pathogens, appears to be sound, but the separation of *Corynebacterium* from *Mycobacterium*, and the introduction of the family Corynebacteriaceae into the Eubacteriales is unjustifiable, in view of the close morphological resemblance between the type species of the two genera (Bisset, 1949). The present genus *Nocardia* is heterogeneous; Umbreit (1939) divides the genus (alternatively called *Proactinomyces*) into  $\alpha$  and  $\beta$  forms, the former resembling corynebacteria and the latter forming a stable mycelium, but without spores. Umbreit suggested that the acid-fast

and non-acid-fast  $\alpha$ -*Proactinomyces* might be related to *Mycobacterium* and *Corynebacterium* respectively, and also related, via the  $\beta$ -*Proactinomyces*, to *Streptomyces*. Jensen (1934) suggested a similar relationship, regarding the various intermediate forms as evidence that these genera form a natural group.

Biochemically the streptomyces, actinomyces, mycobacteria and corynebacteria are well defined, whereas the nocardia, as at present defined, are heterogeneous, but can be divided into two rather ill-defined sub-groups, one of which resembles the mycobacteria in being non-proteolytic, and failing to produce acid from sugars. The second group resembles the streptomyces, being proteolytic and able to hydrolyse starch. It is probable that these two sub-groups are not closely related.

#### MATERIALS AND TECHNIQUE

Morphological examinations were made by Gram's method and by Robinow's (1945) tannic acid violet stain for cell-walls. Preparations made by the latter method are of such value in the elucidation of the morphology of bacteria, and the technique is so simple to perform, that any morphological study which fails to make use of it, and thus fails to determine whether the organism described is composed of one cell or many, must be regarded as incomplete.

The following strains of bacteria were examined:

*Pathogenic actinomyces.* Eight newly isolated strains, two from human, and six from bovine infections, all were anaerobic on isolation.

*Other parasitic organisms.* Two non-sporing, actinomyces-like organisms from the human mouth, aerobic and apparently non-pathogenic. Four sporing actinomyces (*Streptomyces*) from the healthy mouths of laboratory animals.

*Soil 'diphtheroids' and nocardia.* Thirty strains of these organisms were isolated from soil, and six strains of Gray & Thornton's (1928) *Proactinomyces* were obtained from the National Collection of Type Cultures.

*Streptomyces.* Thirty-one strains were isolated from soil and water, in addition to the four from animal sources mentioned above.

#### OBSERVATIONS

The pathogenic actinomyces were grown anaerobically upon nutrient agar containing 1 % glucose. The colonies were adherent to the medium and consisted of radiating filaments (Pl. 1, fig. 1), in which little or no branching was observed. This contrasted strongly with the appearance of the aerobic, sporing organisms (*Streptomyces*) which branched very obviously (Pl. 1, fig. 2), and in which the branched condition of the component cells, as described by Klieneberger-Nobel (1947), was readily seen.

In smears stained by tannic acid violet the pathogenic forms showed occasional, short branches, which soon became separated from the main stem. This type of branching is more accurately described as budding (Pl. 1, figs. 3, 4). The strains from human and bovine sources, including the two aerobic strains from the normal human mouth, were alike in this respect, but differed in that the filaments of the human strains were invariably much longer than

in the bovine strains. The component cells were also of great length in the human strains (Pl. 1, figs. 5, 6). The constancy of this difference throughout several weeks of culture suggests that this species may be subdivided into human and bovine types analogous to those of *M. tuberculosis*. On sub-culture the anaerobic character progressively diminished, and disappeared after a few weeks. The colonies also tended to become less adherent.

A more complete examination of the morphology of the sporing strains was not attempted, as these organisms have already been competently described (Klieneberger-Nobel, 1947).

The soil bacteria and proactinomyces were of two major morphological groups. Those corresponding to the general description of 'soil diphtheroids', branched only occasionally and the branch was separated from the parent cell (Pl. 1, fig. 7). In some strains the cell-wall was highly irregular, and produced a fallacious appearance of branching in a Gram-stained film (Pl. 1, fig. 8). These bacteria were unicellular, and quite distinct from the true corynebacteria, which are multicellular (Bisset, 1949). In the early stages of growth all the strains of this group produced an unstable mycelium which later fragmented into bacillary forms. Surface colonies on Lemco agar were pinkish, glistening and easily emulsified.

The second type consisted of more filamentous organisms, and formed surface colonies on Lemco agar which were white, waxy and difficult to emulsify. The filaments were frequently branched and were composed of numerous very short cells. They bore a general resemblance to the branching mycobacteria described by Brieger & Fell (1945). As with all other forms observed, except streptomyces, the mature branch was separated from the parent filament, and the component cell was not branched (Pl. 1, fig. 9). By Gram's method, the shrunken cell contents formed Gram-positive granules in the Gram-negative filament (Pl. 1, fig. 10).

## DISCUSSION

We have attempted to discover a series of morphological criteria by which the various types of branched bacteria might be classified. There is no real justification for the present habit of confining morphological examination to Gram-stained, heat-fixed preparations, especially as the tannic acid violet technique, which reveals the cell boundaries, is simpler than Gram's method to perform.

We consider that the sporing aerobic *Streptomyces* are entirely distinct from the other organisms described, and propose a separate Order, Streptomycetales, for them, retaining the Order Actinomycetales to include the remainder of the groups under discussion. The character of acid-fastness is widely distributed in both orders, and, unless pronounced, is not of great importance. Bacteria of the 'soil diphtheroid' type have not, in the past, been well described, and individual species have been assigned to various genera, especially *Corynebacterium*, *Mycobacterium* and *Nocardia*. A new genus, *Jensenia*, is proposed to include the aerobic, unicellular bacteria of this type.

The two orders are defined as follows.



## Order STREPTOMYCETALES

Filamentous, branching bacteria. (Their position in the *Schizomycetes* is assumed but is questionable.) Mature branch continuous with parent stem. Cell branched. Spores formed (Fig. 1).

There is one family, Streptomycetaceae, consisting of two genera, *Streptomyces* and *Micromonospora*, defined as in Bergey (1948).

Fig. 1. *Streptomyces*.

(a)



(b)

Fig. 2. (a) *Actinomyces*  
(b) *Jensenia*.

(a)



(b)

Fig. 3. (a) *Nocardia*, (b) *Mycobacterium* and *Corynebacterium*.

## Order ACTINOMYCETALES

Filaments or rods, sometimes branched. The mature branch is divided from the parent filament by a partition. Cell unbranched. Spores not formed.

Two families are described, which are distinguished by the length of the cells comprising the bacillus or filament.

## Family I. ACTINOMYCETACEAE

Cells several times as long as broad. Individual bacillus unicellular.

Genus A. *Actinomyces*

Filamentous, often anaerobic or microaerophilic. Animal parasites and pathogens (Fig. 2a).

Genus B. *Jensenia*

'Soil diphtheroids'. Aerobic, short, often irregular in shape, occasionally branched (Fig. 2b).

## Family II. MYCOBACTERIACEAE

Breadth of cells approximately the same as length. Individual bacillus is multicellular.

Genus C. *Mycobacterium*

Strongly acid-fast. Occasionally branched (Fig. 3b).

Genus D. *Nocardia*

Slightly or non-acid-fast. Filamentous, often branched (Fig. 3a).

Genus E. *Corynebacterium*

Non-acid-fast. Short, unbranched. Animal parasites and pathogens (Fig. 3b).





Figs. 1-10

In this classification no effort has been made to accommodate the various genera (e.g. *Erysipelothrix*) of which adequate morphological descriptions are not available, and which we have not examined. We consider that the criteria of types of branching and types of cell are sufficiently clear-cut to make allocation to families relatively easy, once these genera are properly described.

The greatest defect of the proposed system is that the characters defining the various groups are relative, that is to say, they may sometimes lie within the limits of variability of a single species at various ages and conditions of culture. Very small corynebacteria may often consist of a single cell; aged cultures of actinomyces are sometimes composed of multicellular, bacillary forms; in the initial stages, all branches may be alike, and so forth. Nevertheless, we consider that the scheme approximates more closely to the true relationship of the bacteria, and is more readily applicable to the description of new genera and species, and the rearrangement of those already recorded, than those now in general use.

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#### EXPLANATION OF PLATE

- Fig. 1. *Actinomyces bovis*, edge of colony on agar, unstained.  $\times 350$ .
- Fig. 2. *Streptomyces* sp., edge of colony on agar, unstained.  $\times 350$ .
- Figs. 3, 4. *A. bovis*, budding and separation of branches. Tannic acid violet.  $\times 3000$ .
- Fig. 5. *A. bovis*, bovine type, typical short filaments. Tannic acid violet.  $\times 3000$ .
- Fig. 6. *A. bovis*, human type, typical long filaments. Tannic acid violet.  $\times 3000$ .
- Fig. 7. *Jensenia* sp. bacillary forms and small branch. Tannic acid violet.  $\times 3000$ .
- Fig. 8. *Jensenia* sp. showing irregular outline. Tannic acid violet.  $\times 3000$ .
- Fig. 9. *Nocardia* sp. multicellular, branched filament. Tannic acid violet.  $\times 3000$ .
- Fig. 10. *Nocardia* sp. granular appearance by Gram's stain. Granules are shrunken cell contents.  $\times 3000$ .

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## The Toxicity of Small Concentrations of Cystine to Acid-producing Bacteria

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**SUMMARY:** None of the various methods proposed for the microbiological assay of cystine is entirely satisfactory. *Leuconostoc mesenteroides* P-60 appeared to be the most suitable micro-organism, but assays were nevertheless variable and, occasionally, completely unreliable. A toxic effect characterized all assays to some extent, but no satisfactory explanation of its occurrence can be advanced.

As yet no entirely satisfactory microbiological method for the assay of cystine has been reported. Assays are described, using *Lactobacillus arabinosus*, by Shankman, Dunn & Rubin (1943) and by Barton-Wright (1946), but the absolute requirement of cystine by *L. arabinosus* has not been confirmed by Riesen, Spengler, Robblee, Hanks & Elvehjem (1947) or by work in this laboratory (unpublished data). With oxidized-peptone medium, Lyman, Moseley, Wood & Hale (1947) found that, though smooth and regular standard curves could be obtained for methionine, tyrosine and tryptophan on media in which most of the amino-acids required were supplied by oxidized-peptone, standard curves for cystine using the same organism (*L. casei*  $\epsilon$ , *L. helveticus*) proved to be irregular and inconsistent. Similar conclusions about the non-reproducibility of standard curves for cystine with this organism were reached by Shankman *et al.* (1943). Dunn, Shankman, Camien, Frankl & Rockland (1944) stated that cystine could be determined by *Leuconostoc mesenteroides* P-60, and Riesen *et al.* (1947) concluded that this organism is the most suitable for the determination of this amino-acid.

Assays of cystine carried out in this laboratory with *Leuconostoc mesenteroides* P-60 on oxidized-peptone medium or a known mixture of amino-acids yielded standard curves which varied in range of titre from assay to assay. No such effects were observed when methionine or tyrosine were assayed using the same organism and similar media, and the assay range, as shown by higher titres, was greater in either case than in assays of cystine under the same conditions. With regard to media for cystine assays, oxidized-peptone is to be preferred since blanks are usually lower than those obtained on chemically defined media (amino-acid mixtures). Riesen *et al.* (1947), also, have demonstrated the superiority of oxidized-peptone medium for the assay of cystine, using several organisms for which cystine is said to be essential.

There is a further complication due to an apparent toxic effect, characterized by a slight break in the assay curve but also shown by a clumping of the bacteria. Fig. 1 shows a standard curve for cystine with rather better than average titres, compared with a normal standard curve for methionine. The same oxidized-peptone medium was used in each case, with the appropriate omissions of methionine and cystine respectively. The clumping of the bacteria results in

the formation of a solid cone the base of which clings to the bottom of the test-tube, whilst the mass of bacteria swirl round and round the tube when the latter is shaken with a rotatory movement. With a healthy culture of cells, this same movement would disperse the cells evenly throughout the tube. Several possibilities which might explain the phenomenon were investigated as follows.

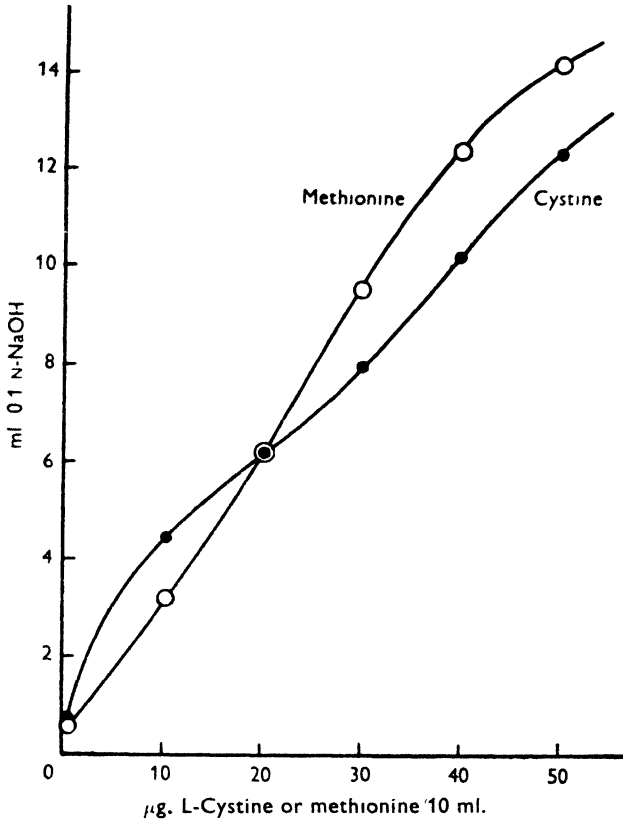


Fig. 1. Dose-response curves of *Leuconostoc mesenteroides* P-60, produced by additions of DL-methionine and DL-cystine, respectively, to the same (oxidized-peptone) medium.

*Cysteic acid.* The presence of this substance might account for toxicity on oxidized-peptone medium but would not explain the effect in the chemically defined medium. Further, cysteic acid is present during assays of methionine and tyrosine using oxidized-peptone medium without harmful effects to the bacteria.

*D-Cystine.* The toxicity might result from the D-isomer of the amino-acid, since DL-cystine was used in preparing the standard solution. Comparable assays carried out under exactly the same conditions and at the same time showed little difference in the form of standard curve or in the assay range, whether the natural isomer (L) or the racemic mixture (DL) as in the synthetic product was used. Since only the L-isomer is active, standard solutions of DL-cystine were used in twice the concentration of those of L-cystine for comparable assays. In both cases the full toxic effect was observed.

*Deficiency due to lack of SH-groups.* Though similar cases of bacterial clumping with *Leuconostoc mesenteroides* on nutritionally complete media have been observed in this laboratory (unpublished data), it was considered desirable to test whether a deficiency of SH-groups might be operative. The argument can legitimately be raised since, in the assay of methionine, a concentration of 100  $\mu\text{g.}$  of L-cystine/10 ml. of medium is present, whereas the highest concentration of the standard cystine corresponds to a concentration of only half this amount. The addition of thiolacetic acid in amount equivalent to that of L-cystine (0.1 g./l.) contained in the medium for methionine assay, however, produced only a very slight lowering of the titres. No diminution in toxicity was observed.

Finally the possibility cannot be entirely excluded that bacterial clumping itself might be the cause rather than the result of the apparent toxicity.

Bacterial clumping was also observed in assay tubes of media containing protein-hydrolysates as well as in the standard tubes. It takes place after incubation at 37° for 16 hr. It would appear, *prima facie*, that L- and DL-cystine are toxic to *Leuconostoc mesenteroides* P-60 (and possibly to other lactic acid-producing bacilli) when added in small amounts (e.g. 0–50  $\mu\text{g.}$  /10ml. for L-cystine) to media otherwise nutritionally adequate for the organism. This toxicity is independent of the need of cystine for growth and probably accounts for the variability in assay range in the microbiological assay of cystine. Caution in accepting figures resulting from one or two assays only would appear to be necessary.

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# The Synthesis of Polysaccharides by Bacteria Isolated from Soil

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**SUMMARY:** Bacteria capable of synthesizing polysaccharides are present in various agricultural, moorland and forest soils, and may form from 5–16% of the viable bacterial population, as estimated by the plate-dilution counting technique. Chemical examination of the polysaccharides synthesized distinguished four types: levans, glucose-uronic acids, glucose-mannose-uronic acids and glucose-mannose-rhamnose-uronic acids. Bacteria synthesizing these polysaccharides are well distributed in the soils studied, those forming the first two types being by far the most common. There is no clear relationship between morphology of the organisms and type of polysaccharide synthesized.

It has been suggested (Norman, 1942) that the uronide carbon of soil organic matter may be in part derived from microbial polysaccharides. Indirect evidence in support of this hypothesis has been brought forward by Fuller (1947), and Forsyth (1947) isolated from soils a definite polysaccharide fraction which contained uronic acid units. Although an important role in determining the physical structure of soils has been suggested for bacterial polysaccharides (Martin, 1946), little has been done in isolating the bacteria in soil which are capable of synthesizing polysaccharides, or in determining the nature of the polysaccharides produced. Martin (1945) has made a start in this direction. He found, using a medium containing egg-albumin, or sodium nitrate and glucose or sucrose, and pouring plates from a high dilution, that nearly every plate contained from one to five slimy colonies. The majority of the organisms forming these colonies were capsulated. We have surveyed different soils (agricultural, moorland, and forest) to determine (*a*) the presence, and if possible the proportion, of gum-producing bacteria, and (*b*) the nature of the polysaccharides produced by these organisms.

## METHODS

**Bacteriological methods.** The medium used throughout this work was that of Cooper & Preston (1937), called by them Medium II. For counting purposes the  $\text{CaCO}_3$  which this medium ordinarily contains can be omitted. Soil samples were taken with sterile precautions and plated by the dilution technique of Cutler, Crump & Sandon (1922) on the day the samples were collected, and usually incubated from 3–4 days at 25°. With longer incubation the plates were frequently overgrown by spreading organisms. The sucrose, used in 2% concentration in the medium, was sterilized separately at double strength and 4 ml. added to an equal quantity of double-strength mineral base + agar.

The proportion of slimy colonies was noted and each colony examined for capsulated organisms before inclusion in the count. From among these colonies (developing on the five replicate plates) random isolations were then made on



to Cooper & Preston's solid medium. Pure cultures obtained on this medium were grown for 2-4 days at 25° on four plates of the same medium and the growth harvested for investigation.

**Chemical methods.** The polysaccharides were purified and hydrolysed as described by Forsyth & Webley (1949) and the constituent sugars identified by the paper chromatography technique (Partridge, 1946, 1948; Forsyth, 1948) as follows.

**Fructose.** After hydrolysis with 0.1N-H<sub>2</sub>SO<sub>4</sub> for ½ hr. at 100°, fructose was identified from its *R<sub>F</sub>* values (*n*-butanol, 0.10; phenol, 0.51; *s*-collidine, 0.42), and confirmed by the ketose reaction on spraying the chromatograms with naphthoresorcinol or resorcinol reagent. When fructose was present it was destroyed by dilute acid hydrolysis of another sample of the gum and the residual polysaccharide, if any, reprecipitated with ethanol and subjected to stronger hydrolysis (N-H<sub>2</sub>SO<sub>4</sub> for 4 hr.) to test for other sugars. When fructose was not present, another sample of gum was directly hydrolysed with the N-H<sub>2</sub>SO<sub>4</sub> for the other sugars. Only 10-20 mg. samples were required.

**Glucose.** *R<sub>F</sub>* values: *n*-butanol, 0.07; phenol, 0.39; *s*-collidine, 0.39. That the sugar was an aldohexose was confirmed by resorcinol spray.

**Mannose.** *R<sub>F</sub>* values: *n*-butanol, 0.10; phenol, 0.45; *s*-collidine, 0.46. That the sugar was an aldohexose was confirmed by resorcinol spray.

**Rhamnose.** *R<sub>F</sub>* values: *n*-butanol, 0.22; phenol, 0.59; *s*-collidine, 0.59. That the sugar was a methylpentose was confirmed by naphthoresorcinol and resorcinol spray.

**Uronic acids.** These were detected both by the naphthoresorcinol test on the intact polysaccharide and by spraying chromatograms of the hydrolysate with this reagent. They also give spots close to the starting line with the silver nitrate reagent.

## RESULTS

From Table 1 it will be seen that the polysaccharide-producing bacteria were present in all three types of soil examined. The proportion varied from about 5% of the total viable bacterial flora in the *B*<sub>1</sub> layer of the moorland soil to about 16% in the top 6 in. of the agricultural soil. From the soils listed in Table 1 and from one other agricultural soil 40 strains were isolated at random from the slimy colonies on the plates. Table 2 gives a complete list of the

Table 1. *Gum-producing bacteria in soils*

| Soil sample                                      |                               | Dilution used<br>for plating | Mean count<br>(3-5 plates) |                | Proportion<br>of gum-pro-<br>ducing<br>bacteria<br>(%) |
|--|-------------------------------|------------------------------|----------------------------|----------------|--|
| Location and type                                | Depth                         |                              | Total                      | Gummy colonies |  |
| Moorland (Dinnet)                                | <i>A</i> <sub>2</sub> * layer | 1/2,500                      | 120                        | 13             | 10.8   |
|  | <i>B</i> <sub>1</sub> * layer | 1/2,500                      | 73                         | 5              | 4.5  |
| Broad-leaved wood-<br>land (Dunottar)            | 2-4 in.                       | 1/25,000                     | 98                         | 9              | 9.5  |
|  | 12-15 in.                     | 1/25,000                     | 76                         | 11             | 15   |
| Agricultural 3rd year<br>year grass (Stonehaven) | 0-6 in.                       | 1/250,000                    | 131                        | 21             | 16   |

\**A*<sub>2</sub> = Upper leached layer of the soil (usually grey in colour).

\**B*<sub>1</sub> = Upper layer of less strongly leached sub-soil (usually reddish or yellowish in colour).

Table 2. *The morphological characters of soil bacteria and the constituent sugars of the polysaccharides synthesized by them*

| Soil sample                                |             |                        |   |
|--|-------------|------------------------|---|
| Location and type                          | Depth       | Morphology             | Components of the polysaccharides       |
| Dinnet Moor                                | Layer $A_2$ | Spore former, Group 1  | Fructose                                |
|  |             | Short non-sporing rod  | Glucose, mannose, rhamnose, uronic acid |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Spore former, Group 1  | Fructose                                |
|  | Layer $B_1$ | Spore former, Group 1  | Fructose                                |
|  |             | Spore former, Group 2  | Fructose, glucose, mannose, uronic acid |
|  |             | Short non-sporing rod  | Glucose, mannose, rhamnose, uronic acid |
|  |             | Spore former, Group 1  | Glucose, uronic acid                    |
| Tillicecorthy; agricultural soil           | 12-15 in.   | Spore former, Group 1  | Glucose, uronic acid                    |
|  |             | Spore former, Group 2  | Fructose, glucose, uronic acid          |
|  |             | Spore former, Group 2  | Glucose, mannose, uronic acid           |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
| Dunottar Woods; broad-leaved woodland soil | 2-4 in.     | Spore former, Group 2  | Fructose, glucose, mannose, uronic acid |
|  |             | Short non-sporing rod  | Glucose, mannose, rhamnose, uronic acid |
|  |             | Medium non-sporing rod | Glucose, mannose, uronic acid           |
|  |             | Spore former, Group 2  | Glucose, mannose, uronic acid           |
|  | 12-15 in.   | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Spore former, Group 1  | Fructose                                |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, mannose, rhamnose, uronic acid |
|  |             | Short non-sporing rod  | Fructose, glucose, uronic acid          |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
| Stonehaven; agricultural soil              | 0-6 in.     | Spore former, Group 1  | Fructose                                |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Spore former, Group 1  | Fructose                                |
|  |             | Spore former, Group 1  | Fructose                                |
|  |             | Pleomorphic type       | Fructose                                |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, uronic acid                    |
|  |             | Short non-sporing rod  | Glucose, mannose, rhamnose, uronic acid |
|  |             | Pleomorphic rods       | Glucose, uronic acid                    |
|  |             | Pleomorphic rods       | Glucose, uronic acid                    |
|  |             | Medium non-sporing rod | Fructose, glucose, uronic acid          |
|  |             | Pleomorphic rods       | Glucose, mannose, uronic acid           |
|  |             | Spore former, Group 1  | Glucose, uronic acid                    |
|  |             | Spore former, Group 1  | Fructose                                |
|  |             | Spore former, Group 1  | Fructose                                |

bacteria, their morphological character, and the constituent sugars of the polysaccharides synthesized by each. The spore-forming bacteria have been divided into groups after Smith, Gordon & Clark (1946). The pleomorphic organisms were similar morphologically to those described by Conn & Dimmick (1947) under the genus *Arthrobacter*.

### DISCUSSION

It is apparent from Table 1 that gum-producing bacteria are present in reasonable numbers in all the soils studied, and from Table 3 that the levan and glucose-uronic types of polysaccharides predominate in the soils studied. A glucose-mannose-uronic type is also present. A group of organisms synthesizing a polysaccharide containing rhamnose is moderately prevalent and well distributed. Such a polysaccharide has not hitherto been isolated from a non-pathogenic bacterium.

Table 3. *The different types of polysaccharide gum produced by soil bacteria; distribution among the soils*

The different types of bacterial gums give on hydrolysis the following sugars: *A*, fructose; *B*, fructose, glucose, uronic acid; *C*, fructose, glucose, mannose, uronic acid; *D*, glucose, uronic acid; *E*, glucose, mannose, uronic acid; *F*, glucose, mannose, rhamnose, uronic acid.

| Soil                      | Type of polysaccharide synthesized |          |          |          |          |          | Total |
|---------------------------|------------------------------------|----------|----------|----------|----------|----------|-------|
|                           | <i>A</i>                           | <i>B</i> | <i>C</i> | <i>D</i> | <i>E</i> | <i>F</i> |       |
|                           | No. of isolates                    |          |          |          |          |          |       |
| Dinnet moorland           | 3                                  | 0        | 1        | 3        | 0        | 2        | 9     |
| Dunottar woodland         | 1                                  | 1        | 1        | 6        | 2        | 2        | 13    |
| Stonehaven agricultural   | 6                                  | 1        | 0        | 6        | 1        | 1        | 15    |
| Tilliecorthy agricultural | 0                                  | 1        | 0        | 1        | 1        | 0        | 3     |
| Totals                    | 10                                 | 3        | 2        | 16       | 4        | 5        | 40    |

It is known that the Group 1 bacilli of Smith *et al.* (1946) produce levans (Harrison, Tarr & Hibbert, 1930; Challinor, Haworth & Hirst, 1934; Lyne, Peat & Stacey, 1940; Forsyth & Webley, 1949). Group 2 bacilli produce glucose-uronic and glucose-mannose-uronic types (Forsyth & Webley, 1949). Soil organisms such as *Rhizobium* spp. and *Azotobacter* spp. (Cooper, Daker & Stacey, 1938) and possibly the *Cytophaga* group (Walker & Warren, 1938) also produce polysaccharides of the glucose-uronic type. Martin (1945) has isolated from soil non-spore-forming rods which synthesize polysaccharides of this type.

Table 4. *Morphology of soil bacteria and type of polysaccharide synthesized*

| Morphological group     | Type of polysaccharide* synthesized |          |          |          |          |          | Total |
|-------------------------|-------------------------------------|----------|----------|----------|----------|----------|-------|
|                         | <i>A</i>                            | <i>B</i> | <i>C</i> | <i>D</i> | <i>E</i> | <i>F</i> |       |
| Spore-former Group 1    | 9                                   | 0        | 0        | 3        | 0        | 0        | 12    |
| Spore-former, Group 2   | 0                                   | 1        | 2        | 0        | 2        | 0        | 5     |
| Short non-sporing rods  | 0                                   | 1        | 0        | 11       | 0        | 5        | 17    |
| Medium non-sporing rods | 0                                   | 1        | 0        | 0        | 1        | 0        | 2     |
| Pleomorphic rods        | 1                                   | 0        | 0        | 2        | 1        | 0        | 4     |

\* As in Table 3.

When a comparison is made of the morphological characteristics and the type of polysaccharide synthesized by the soil bacteria, it is seen (Table 4) that there is no clear correlation between morphology and type of polysaccharide synthesized; different types of polysaccharide are produced by members of a given morphological group. The results for the spore-formers are in close agreement, as regards the type of polysaccharide produced within each group, with the findings of Forsyth & Webley (1949) for type cultures of species of the genus *Bacillus*. The predominant group of short non-spore-forming rods produce gums chiefly of the glucose-uronic type.

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## Antibiotics produced by *Bacillus licheniformis*

### 1. A Practical Chemically Defined Medium for Production of Licheniformin

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**SUMMARY:** The production of licheniformin-like antibacterial activity in culture by a single strain of *Bacillus licheniformis* required neutral or alkaline conditions, conveniently attained by the use of lactate rather than glucose as a source of carbon in a chemically defined medium.

When the medium contained initially about 0.04–0.10% nitrogen, supplied as asparagine or as ammonium lactate, and the cultures, harvested after 4–9 days at 37°, were sterilized by bringing to pH 2.5 and autoclaving, the inhibitory dilution against a test strain of *Mycobacterium phlei* was 1/160 or greater.

Fluid from cultures in a chemically defined medium, containing 0.06 M ammonium lactate and 0.05 M sodium lactate, inhibited the test organism at a dilution of 1/200–1200 (geometric mean, 530) in 44 consecutive batches of 100–200 l. culture fluid produced by incubation of cultures in shallow layers. The pH value of the harvested fluid was about 9 and the antibiotic material was partly bound by the cells. It was largely freed by adjustment to pH 2.5.

When amino-acids were added to the medium either as a mixture of known amino-acids, or as a casein hydrolysate, the maximum titre was attained earlier, but with no significant change in its value. A similar result was obtained with yeast extract added alone or with casein hydrolysate.

Licheniformin is an antibiotic whose production by a strain of *Bacillus licheniformis* was first reported by Callow & Hart (1946). The isolation of highly concentrated material and its chemical and biological properties were described briefly by Callow, Glover & Hart (1947), and in more detail by Callow, Glover, Hart & Hills (1947). Cultures with similar biological and chemical properties were obtained only from those spore-bearing aerobic organisms (11 of 81 cultures examined) which were identified as *B. licheniformis* by the criteria of Gibson (1944).

The present communication describes the development of a chemically defined medium for the production of active culture fluids. A preliminary communication indicated the reasons for adopting the formula now in use (Hart & Hills, 1947) which has lactate as main source of carbon and ammonia as source of nitrogen. The efficacy of such simple nutrients was not foreseen at the beginning of the work, since at that time aerobic spore-bearers were reported either as failing completely to produce antibiotic from such sources, as in subtilin production (Jansen & Hirschmann, 1944) or as producing relatively low yields in comparison with those from more complex media, as in tyrothricin production (Lewis, Dimick & Feustel, 1945). Hence, in spite of the desirability of using a chemically defined medium to facilitate isolation of the active material free from impurities introduced in the medium, it was considered expedient to examine antibiotic production in a complex medium of controllable

composition. A suitable medium for this purpose was the CCY medium of Gladstone & Fildes (1940) in which the nitrogen is supplied as the amino-acids of an acid hydrolysate of casein, supplemented with a tryptic digest of the same protein to supply tryptophan. Other water-soluble growth factors are provided by a boiled yeast extract.

#### METHODS

The techniques were in general those of Callow *et al.* (1947), modified as described below.

**Cultures.** The organism used throughout was the Hampstead strain of *B. licheniformis*, now N.C.T.C. 7072, maintained on glycerol asparagine agar. Experimental media were inoculated from a liquid glucose asparagine culture as described by Callow *et al.* (1947). In addition, spores from the original culture, as received from Hampstead, were stored in the dried state by washing off in distilled water, heating for 90 min. at 60° to destroy vegetative forms, and drying in 0.05 ml. amounts in 10 × 45 mm. tubes over P<sub>2</sub>O<sub>5</sub> at a pressure of 0.05 mm. Hg. The tubes of dried spores were evacuated again in test-tubes, which were sealed and stored at 2°.

For convenience, spore suspensions were used in the inoculation of the medium finally adopted for routine production. They were prepared from the tubes of dried spores, which were incubated at 37° for 6 hr. in tryptic beef broth; 0.5 ml. was then seeded into asparagine glucose liquid medium without Mn (Callow *et al.* 1947), and incubated overnight at 37°. One and a half ml. of this culture was seeded on to 30 ml. quantities of solid maintenance medium (Callow *et al.* 1947) in 8 oz. medical flats. Complete sporulation occurred in 7 days at 37°. The spore suspensions were standardized by the method of Callow *et al.* (1947).

**Culture media.** The following media were tested for production of antibiotic: the CCY medium of Gladstone & Fildes (1940), the glucose + asparagine medium of Callow & Hart (1946), the two 'growth' media of Callow *et al.* (1947), and modifications of these.

**Conditions of incubation.** All cultures were incubated for varying periods at 37°. Most of the work was done with stagnant cultures of 15 ml. of medium in 50 ml. conical flasks, 50 ml. in 250 ml. conical flasks or 1 l. in rectangular enamelled-steel trays, approx. 25 × 40 cm. In each case the average depth of medium was about 1 cm. Separate flasks or trays, or a pool of replicates were used for each determination, since it was found that disturbance of the pellicle by sampling led to irregular results on continued incubation. In a few tests 15 ml. cultures were shaken in 50 ml. flasks at 120 cycles/min., the amplitude being 2.5 cm.

**Precision of the method of assay.** Experience with the method as described by Callow *et al.* (1947) suggested that even two-fold differences between single assays could not be regarded as significant. This was confirmed by statistical analysis of the results for 20 consecutive 150 l. batches of culture. Assays were carried out in duplicate, or sometimes triplicate, on samples of culture which had been subjected to three different treatments. Bartlett's test showed no

significant heterogeneity of variance of the log potency between the three treatments ( $P > 0.25$ ) and there was no significant correlation ( $r = +0.085$ ;  $P > 0.1$ ) between the log potency and its standard error within the observed range of end-points (dilutions of 1/300–1/1500). A pooled estimate of the variance, based on the 71 degrees of freedom available from the 60 samples, corresponded to a standard error of 0.11 for the log potency and an insignificant difference ( $P = 0.95$ ) for observations in the ratio less than 2.0. Since this ratio was only diminished to 1.5 for the difference between the means of triplicates, it was usually expedient to save labour on replication of assays in favour of confirmation of results by total repetition of experiments. Crucial tests were, however, planned in a form providing internal evidence of accuracy. Geometric rather than arithmetic means were used in pooling the results of assays, in view of the uniformity of variance of the log potency.

## RESULTS

### *Media with casein hydrolysates as source of nitrogen*

Since the original medium of Callow & Hart (1946), with asparagine as source of N, contained 3% glucose, modifications of CCY medium with this concentration of sugar were compared with normal CCY medium containing 0.65% sodium lactate (58 mM), and a modification without either lactate or glucose. Table 1 shows that, although it was possible to get moderate titres at 6 days

Table 1. *The effect of carbon source on licheniformin production in CCY medium compared with glucose asparagine medium*

| Medium                      | Licheniformin<br>units/ml. at (days) |    |     | pH at (days) |     |     |
|-----------------------------|--------------------------------------|----|-----|--------------|-----|-----|
|                             | 2                                    | 4  | 6   | 2            | 4   | 6   |
| CCY, no lactate             | 101                                  | 40 | 20  | 8.5          | 9.1 | 9.3 |
| CCY + 58 mM lactate         | 80                                   | 40 | 20  | 8.7          | 9.3 | 9.4 |
| CCY + 150 mM glucose        | 20                                   | 20 | 20  | 5.7          | 7.5 | 8.3 |
| Asparagine + 150 mM glucose | 23                                   | 80 | 101 | 6.9          | 8.0 | 7.2 |

with the medium of Callow & Hart (1946), CCY medium with the same glucose concentration produced no antibiotic within this period, whereas in the absence of glucose moderate titres were reached within 2 days, declining on further incubation. It appeared from the pH changes that a slightly alkaline reaction was an essential but not sufficient condition for antibiotic formation. Excessive alkalinity, on the other hand, was to be avoided.

In other experiments titres as high as 320 units/ml. (i.e. cultures inhibiting at a dilution of 1/320) were reached as early as 18 hr. and were sometimes maintained for as long as 4 days after inoculation. Initial acidity delayed or prevented the attainment of a high titre even when good growth occurred. In media with and without lactate, the omission of glycerophosphate buffer or its replacement by an equivalent amount of phosphate had no effect (Table 2). In all cases a pH value of about 9 was reached by the third day and subsequently the titre fell considerably.

Although moderate titres were reached in CCY medium, this was never used for routine production of antibiotic, since observations on the conditions of antibiotic formation in that medium led to modifications of chemically defined media that gave consistently higher titres. Hence, further modification of CCY medium was not undertaken.

Table 2. *The effect of buffer on licheniformin production in CCY media*

| Buffer (33 mM)   | Lactate concentration (mM) | Licheniformin units/ml. at (days) |     |
|------------------|----------------------------|-----------------------------------|-----|
|                  |                            | 3                                 | 6   |
| None             | 0                          | 225                               | 112 |
|                  | 58                         | 225                               | 80  |
| Phosphate        | 0                          | 190                               | 80  |
|                  | 58                         | 112                               | 80  |
| Glycerophosphate | 0                          | 160                               | 66  |
|                  | 58                         | 225                               | 55  |

*Media with asparagine as a source of nitrogen*

The glucose asparagine medium of Callow & Hart (1946) had a number of disadvantages due to the glucose. Thus the glucose concentration was critical and dependent on the type of vessel used for incubation. With 2 % glucose, 50 ml. of medium in 250 ml. conical flasks gave the optimum titre of 320 units/ml., but 250 ml. medium in cylindrical flasks of about 18 cm. diam. gave more variable and, on the whole, lower titres, although the average depth of medium in both cases was about 1 cm. Glucose also gave highly viscous culture fluids and tough pellicles, to which the active material was bound, and from which it was difficult to isolate.

Table 3. *The effect of replacing glucose by lactate in media with asparagine as source of nitrogen*

| Source of carbon | Concentration (mM) | Licheniformin units/ml. at (days) |     |     |     | pH at (days) |     |     |     |
|------------------|--------------------|-----------------------------------|-----|-----|-----|--------------|-----|-----|-----|
|                  |                    | 2                                 | 3   | 4   | 6   | 2            | 3   | 4   | 6   |
| Lactate          | 16.7               | 225                               | 225 | 160 | 160 | 8.8          | 9.0 | 9.0 | 9.0 |
| Lactate          | 33.3               | 160                               | 80  | 320 | 320 | 8.8          | 9.0 | 9.0 | 9.0 |
| Lactate          | 67                 | 160                               | 160 | 160 | 450 | 8.8          | 9.0 | 9.0 | 9.0 |
| Lactate          | 133                | 80                                | 160 | 160 | 320 | 8.8          | 9.0 | 9.2 | 9.0 |
| Lactate          | 250                | 14                                | —   | 20  | 14  | 9.0          | —   | 9.5 | 9.3 |
| Glucose          | 110                | 20                                | 160 | 160 | 225 | 6.5          | 7.0 | 7.4 | 8.6 |

Since (i) the maximum titre was not reached in this medium until after the initial acid fermentation had ceased and the medium had either regained neutrality or had even become alkaline, and (ii) the maximum titre was reached earlier in those modifications of the CCY medium which never became acid, the effect of replacing glucose by lactate was tested (Table 3). On the basis of this, and similar experiments, it appeared that the maximum titre was reached



earlier with low concentrations of lactate but the best titres were reached in about 6 days with about 0.75 % sodium lactate (67 mM). The lactate concentration, however, was not critical and only in high concentrations, roughly equivalent in total carbon to the glucose previously used, were poor titres observed. Similar results were observed with 15, 50 and 1000 ml. volumes of medium provided the average depth was about the same; variations in this depth from 5 to 15 mm. merely delayed the appearance of maximum activity for periods up to 24 hr.

*Media with ammonia as source of nitrogen*

Although replacing the unsatisfactory media containing high glucose concentrations, the asparagine lactate medium was still not suitable for large-scale production because of difficulty in obtaining adequate supplies of asparagine. Replacement of asparagine by a mixture of ammonium chloride and sulphate, as used by Fildes (1938), gave low titres: 40–80 units/ml. after

Table 4. *Licheniformin production and pH changes with ammonia as source of nitrogen and lactate as source of carbon*

| Lactate<br>(mM) | Ammonia*<br>(mM) | Licheniformin<br>units/ml. at (days) |    |     | pH at (days) |     |     |
|-----------------|------------------|--------------------------------------|----|-----|--------------|-----|-----|
|                 |                  | 1                                    | 2  | 4   | 1            | 2   | 4   |
| 33              | 17               | 40                                   | 80 | 80  | 8.0          | 8.0 | 9.2 |
| 67              | 33               | 40                                   | 80 | 160 | 8.2          | 8.8 | 9.4 |
| 133             | 67               | 20                                   | 80 | 160 | 8.2          | 8.8 | 9.2 |
| 267             | 133              | 0                                    | 0  | 40  | 7.6          | 8.4 | 8.6 |

\* The ammonia was added as an equinormal mixture of thrice recrystallized ammonium chloride and sulphate.

2–6 days with 25–50 mM lactate. The nitrogen content (17 mN), however, was less than 25 % that of the asparagine medium. When increasing the concentration of ammonium salts it was necessary to increase the concentration of metabolizable anions (as in penicillin production on chemically defined media; Jarvis & Johnson, 1947), in order to prevent the development of acidity which could not be adequately controlled by an increased buffer capacity. Titres were thus increased to 160 units/ml. at 4 days with 33–67 mM ammonia and 67–133 mM lactate (Table 4). There was little activity with 133 mM ammonia and 267 mM lactate, but this was not due to adverse pH changes, since development of alkalinity was little delayed compared with that in cultures in less concentrated media. Similar titres were reached 24–48 hr. earlier in shaken cultures and even higher titres, up to 640–1280 units/ml., when the ammonium salts were replaced by a commercially available solution of ammonium lactate. The medium could also be made up with lactic acid (British Pharmacopoeia standard, approx. 12 N) neutralized partly with NaOH and partly with ammonia; but this method was inconvenient, owing to the need for boiling the medium at an alkaline pH to hydrolyse lactide, and a variable decrease in pH value which occurred on autoclaving. This method was therefore abandoned

owing to the uncertain loss of ammonia and the need for a final adjustment of pH under aseptic conditions. It served, however, to show, using 1 l. quantities of medium in trays, that the amount of ammonia (sp. gr. 0.880) could be 2, 3 or 4 ml. giving 30, 45 or 60 mM  $\text{NH}_4^+$ , and the initial pH could be 6.4, 7.0 or 7.5, with little effect on the titre at 7 days (Table 5).

Table 5. *The effect of initial pH and ammonia content of media*

All media contained 10 ml. lactic acid (B.P.)/l.

| Ammonia<br>(sp.gr. 0.880)<br>(ml./l.) | Initial<br>pH | Licheniformin<br>units/ml. at (days) |     |      |
|---------------------------------------|---------------|--------------------------------------|-----|------|
|                                       |               | 6                                    | 7   | 11   |
| 2                                     | 6.4           | 600                                  | 300 | 200  |
|                                       | 7.0           | 250                                  | 400 | 200  |
|                                       | 7.5           | 50                                   | 500 | 400  |
| 3                                     | 6.4           | 500                                  | 500 | 600  |
|                                       | 7.0           | 300                                  | 400 | 500  |
|                                       | 7.5           | 20                                   | 300 | 1000 |
| 4                                     | 6.4           | 400                                  | 600 | 600  |
|                                       | 7.0           | 400                                  | 500 | 600  |
|                                       | 7.5           | 40                                   | 800 | 400  |

This medium was made up most conveniently with commercial 50 % ammonium lactate and 50 % sodium lactate, using 10 ml./l. The initial pH 7.0–7.2 fell to 6.5–6.7 after autoclaving 15 min. at 15 lb./sq. in. Analysis showed about 110 mM lactate (by the method of Friedemann & Graesser, 1933) and 60 mM ammonia, after autoclaving. In one experiment triplicate daily assays at 4–9 days showed a maximum titre of 1100 units/ml. at 5 days, but this hardly differed significantly ( $P \approx 0.95$ ) from the lowest assays of 680 units/ml. observed at 4 and 9 days. The standard error of the log potency was 0.108, identical with the pooled estimate observed later on 20 consecutive batches of culture fluid.

#### *Effects of amino-acids and other growth factors*

The inclusion of 0.15 % acid-hydrolysate or tryptic digest of casein in the basal ammonium sodium lactate medium stimulated growth and gave a markedly raised titre during the first 2–4 days. The maximum titre was usually attained during this period (Table 6), but was not significantly different from that in the control flasks at 6–7 days. This effect was not due merely to increased available nitrogen, because the substitution of an equivalent amount of nitrogen as ammonium lactate did not give an early high titre. A known mixture of amino-acids behaved similarly to the casein hydrolysate. The addition of yeast extract, alone, or with tryptic casein also stimulated growth and gave high titres in 24–48 hr.

The addition of purines and pyrimidines gave a maximum titre in 4 days. A mixture of certain growth factors was inhibitory, viz. biotin, 0.005  $\mu\text{M}$ ; Ca pantothenate, riboflavin and aneurin, 1  $\mu\text{M}$ ; oleic acid and pyridoxin, 2  $\mu\text{M}$ ; nicotinamide, 5  $\mu\text{M}$ ; haemin, 10  $\mu\text{M}$ ; choline chloride, 20  $\mu\text{M}$ ; and inositol,

100  $\mu\text{M}$ . The inhibition was traced to haemin, which was present at 10  $\mu\text{M}$ . In the absence of the haemin a stimulation of growth was observed with the other substances. Van Heyningen (1948) reported inhibition of growth of *B. licheni-*

Table 6. *Effects of amino-acids and other stimulants of growth*

In all experiments duplicate flasks were assayed for each treatment. The titres quoted are the geometric means for the number of experiments indicated in parentheses.

| Supplement   | Licheniformin, units/ml. (no. exp.) at (days) |             |             |             |             |            |             |
|--|---|-------------|-------------|-------------|-------------|------------|-------------|
|  | 1   | 2           | 3           | 4           | 5           | 6          | 7           |
| Nil  | 0<br>(8)                                      | 80<br>(8)   | 80<br>(8)   | 160<br>(8)  | 800<br>(8)  | 700<br>(2) | 1800<br>(1) |
| Ammonium lactate (17 mm)                             | —   | 20<br>(1)   | —           | 400<br>(1)  | —           | 600<br>(1) | 800<br>(1)  |
| Yeast extract ( $\equiv$ 10 % fresh yeast)           | 900<br>(1)                                    | 450<br>(4)  | 600<br>(3)  | 500<br>(4)  | 400<br>(8)  | 500<br>(8) | 250<br>(2)  |
| Tryptic digest of casein ( $\equiv$ 0.15 % casein)   | 600<br>(1)                                    | 600<br>(4)  | 800<br>(8)  | 600<br>(4)  | 800<br>(8)  | 800<br>(3) | 400<br>(2)  |
| Yeast extract + tryptic digest of casein             | 1800<br>(1)                                   | 1300<br>(3) | 1000<br>(8) | 700<br>(3)  | 500<br>(8)  | 300<br>(1) | —           |
| Acid hydrolysate of casein ( $\equiv$ 0.15 % casein) | —   | 400<br>(1)  | —           | 600<br>(1)  | —           | 600<br>(1) | 150<br>(1)  |
| Amino-acid mixture*                                  | —   | 100<br>(1)  | 1800<br>(1) | 1800<br>(1) | 2500<br>(1) | 500<br>(1) | —           |
| Purines + pyrimidines†                               | —   | 0<br>(1)    | 40<br>(1)   | 900<br>(1)  | 450<br>(1)  | 300<br>(1) | —           |

\* Glycine, alanine, serine and glutamate, 1000  $\mu\text{M}$ ; leucine, isoleucine, norleucine, proline, L-hydroxyproline, phenylalanine, tyrosine, aspartate, L-arginine and lysine, 500  $\mu\text{M}$ ; valine, threonine, L-cystine, methionine and L-histidine, 200  $\mu\text{M}$ ; tryptophan, 100  $\mu\text{M}$ . Concentrations, except where shown otherwise, are of the L-form in DL-mixture. Glutamine, 500  $\mu\text{M}$ , was also present.

† Adenine, guanine, xanthine, cytosine, thymine and uracil, 50  $\mu\text{M}$  each.

*formis* and of a number of other aerobic sporing bacilli by haematin in agar at ten times the concentration we used. The haemin effect in our experiments was rather erratic. In a few cultures, growth was observed after a lag of 3–4 days, whereas replicates remained clear for several weeks. This difference was not associated with variations in the initial pH of the medium.

### *Routine production*

The simple ammonia + lactate medium was used without supplements of yeast extracts or casein hydrolysates. One litre volumes were incubated for 6 days in enamelled steel trays with loose-fitting lids. The pH rose to 8.00–9.75. The cultures were acidified to pH 2.5 with about 8 ml. 12 N-HCl/l. and boiled for 20 min., since Callow & Hart (1946), using glucose asparagine medium, had found that some such treatment was needed to free the antibiotic from the cells. After cooling the liquid rapidly in a 120 gal./hr. milk cooler, the cellular debris was removed in a continuous centrifuge.

In 44 consecutive batches, the potencies of the whole culture lay within a range of 270–1200 units/ml., with a geometric mean of 580. Results as low as 270 units/ml. for some early batches were believed to be due to over-heating of the hot-room, when freshly autoclaved vessels of medium for a succeeding

batch were allowed to cool there, preparatory to inoculation at 37°. When the flasks of medium were allowed to cool before transfer to the hot-room, low results were observed only irregularly and were almost certainly due to variability in the assay. In spite of such irregularities, the potencies of the culture fluids were significantly correlated with those of the whole culture ( $r = +0.675$ ;  $P > 0.999$ ) over the complete series (Fig. 1) though shorter series were not always adequate to show it (Table 7).

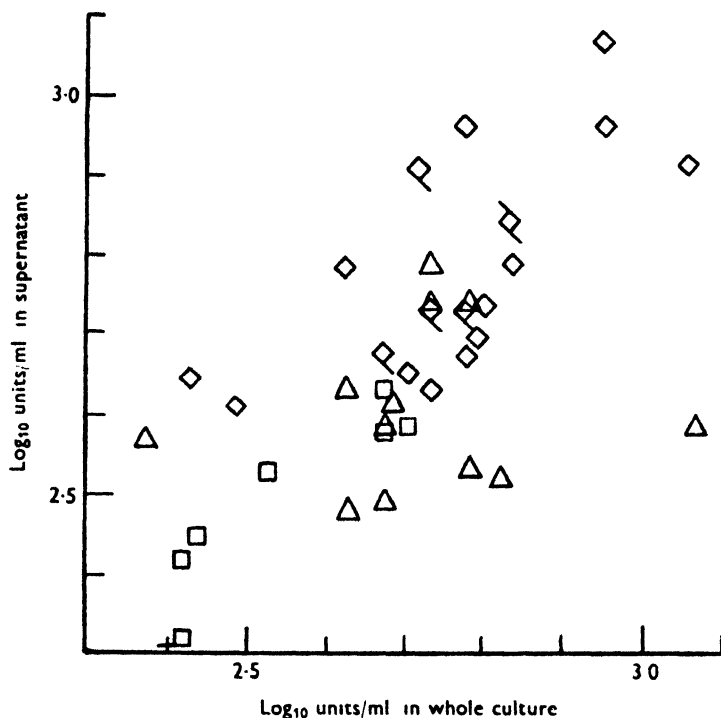


Fig. 1. The recovery of licheniformin in culture fluids. The fluid was removed after boiling 20 min. at pH 2.5; series I, single observations  $\square$ , two identical observations  $\square$ ; series II, cultures also supplying data on removal of fluid at pH 9 and at pH 2.5 without boiling (Table 7)  $\triangle$ ; series III, cultures also supplying data on removal of fluid at pH 2.5 without boiling (Table 7), single observation  $\diamond$ , two identical observations  $\diamond$ , three identical observations  $\diamond$ .

It was found that under these conditions the active material was no longer firmly bound to the cells and over 50 % could be recovered from the supernatant on centrifuging the whole culture at the pH reached during incubation (Table 7). The average loss, however, was highly significant ( $P > 0.999$ ) in the 12 unselected batches on which the determination was carried out. The loss was diminished, but was probably still significant ( $P = 0.98$ ), on centrifuging at pH 2-3 and 36 batches were needed to show significance at this level. Finally, the loss was decreased to 7 %, and was then insignificant ( $P = 0.92$ ) after boiling at this pH and centrifuging either at the same pH or at pH 5. The latter value, pH 5, was frequently convenient, since it was also used for the subsequent adsorption of the active material on charcoal.

## DISCUSSION

The routine medium described is produced cheaply from chemicals available commercially in bulk. Since highly purified chemicals and minimal inocula were not used, we have not established the minimal nutritional requirements of *B. licheniformis*. The mineral requirements, in particular, were not examined,

Table 7. *The recovery of active material in culture fluids*

| Conditions of removal of cells        | No. of batches treated | Correlation coefficient of log potencies of whole culture and supernatant <i>r</i> | Probability of <i>r</i> due to chance <i>P</i> | Percentage recovery |   |   |
|---------------------------------------|------------------------|--|--|---------------------|---|---|
|                                       |                        |  |  | Mean                | Fiducial* limits of mean <i>P</i> =0.95 | Probability of loss* due to chance <i>P</i> |
| At final pH of culture (8.00-9.75)    | 12                     | 0.47   | 0.1  | 56                  | 45-69                                   | 0.001                                       |
| At pH 2.05-3.17                       | 36                     | 0.54   | 0.001  | 88                  | 79-99                                   | 0.021                                       |
| After boiling 20 min. at pH 2.05-3.17 | 44                     | 0.68   | 0.001  | 93                  | 83-103                                  | 0.08  |

\* Since Bartlett's test showed no significant heterogeneity of the variance of the log recovery ( $P=0.26$ ), a pooled estimate was used in calculating the fiducial limits and probability of loss due to chance. This is half the probability of an equal chance deviation without regard to sign.

so that although  $Mn^{++}$  was supplied at  $1.3 \times 10^{-5} M$  as for subtilin production (Jansen & Hirschmann, 1944), the optimal requirement is unknown. Similarly it is not known whether other trace elements are required, such as Zn which is essential for subtilin production (Feeney, Lightbody & Garibaldi, 1947; Feeney & Garibaldi, 1948), and which may have been adequately supplied as an impurity in the chemicals used.

The absence of any increase in the peak titre when the media were supplemented with amino-acids or growth factors, either as pure chemicals or in the form of casein hydrolysates or yeast extracts, contrasts with tyrothricin production (Lewis *et al.* 1945) in which a supplement of tryptone supplying less than one-sixth of the N supplied as ammonium sulphate increased the yield five- to eight-fold when citrate or better, malate, was present. It is, however, to be emphasized that ammonia is likely to serve as an adequate source of N both for growth and antibiotic production by other members of the *Bacillus* group, since it usually supports some growth, which may be improved by 'training' (Knight, 1936). Previous failures have in certain cases been due to the development of adverse conditions, such as pH, during incubation. Thus it is now recognized that ammonia suffices for subtilin production (Feeney, Garibaldi & Humphreys, 1948) in spite of earlier reports to the contrary (Jansen & Hirschmann, 1944). Here, as well as with tyrothricin (Lewis *et al.* 1945), it is essential to avoid acidity by supplying also a metabolizable anion such as citrate. A more elaborate example of pH control using this principle

to maintain, successively, optima both for growth and antibiotic production, is given by the work of Jarvis & Johnson (1947) on penicillin. With bacitracin production (Anker, Johnson, Goldberg & Meleney, 1948) utilization of ammonia does not appear to have been tested; with amino-acids, however, L-glutamate was not consistently replaceable by D-glutamate, asparagine or glycine. The fact that replacement did sometimes occur suggests the possibility that it depended on the initiation of enough growth to cause breakdown of the amino-acids followed by utilization of ammonia.

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## The Influence of Magnesium on Cell Division

### 2. The Effect of Magnesium on the Growth and Cell Division of Various Bacterial Species in Complex Media

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**SUMMARY:** Magnesium is essential for the normal cell division of bacilli in complex media. Under conditions of magnesium deficiency or magnesium excess, cell division is inhibited and filamentous cells may be formed. Under the same conditions there is no appreciable interference with the division of chromatinic bodies. The magnesium requirements of the Gram-positive bacteria are considerably greater than those of the Gram-negative bacteria, possibly because the former incorporate magnesium into the structure of the Gram complex.

In a previous communication (Webb, 1948*a*) it was shown that magnesium was essential for the normal cell division of *Clostridium welchii*. In complex media deficient in ionic magnesium, *Cl. welchii* grew in the form of long filaments, which reverted to cells of normal morphology when subcultured in a medium containing 0.0015 % (w/v) magnesium ion. In extending these studies, the influence of magnesium on the growth and division of various other species of bacteria in complex media has been determined. The results, described here, suggest that magnesium is essential for the normal cell division of all the rod-shaped bacteria studied, but that fundamental differences exist between the magnesium requirements of Gram-positive and Gram-negative organisms.

#### MATERIAL AND METHODS

The majority of the bacteria studied were obtained from the National Collection of Type Cultures and are indicated in the text by the N.C.T.C. 1936 catalogue number following the name of the organism. The remaining cultures were from a collection maintained in this department.

Magnesium analyses were made gravimetrically as previously recorded (Webb, 1948*a*). Although these analyses were tedious and required about 2 g. dry cells for each determination in duplicate, the method was more accurate and more reproducible than colorimetric determinations.

A peptone water medium containing 2 % (w/v) peptone (Evans Medical Supplies, London) was made deficient in ionic magnesium by precipitation of this element as magnesium ammonium phosphate as previously described (Webb, 1948*a*). By this means 94 % of the total magnesium present in the peptone was removed and the final medium contained *c.* 0.00008 % (w/v) Mg.

When Mg-deficient liquid media were solidified with agar, the magnesium present in the agar was utilized by bacteria. Ignition of agar (Ward, Blenkinsop and Co.) gave 1.84 % ash, which contained 2.65 % magnesium. When magnesium, together with other metallic ions ( $K^+$ ,  $Na^+$ ,  $Ca^{++}$ ,  $Fe^{+++}$ ,  $Sn^{++}$ ), was removed by electro dialysis at 45°, the agar completely lost its setting properties.

Tin, iron, calcium and magnesium were completely removed when 0.1 N hydrochloric acid (2 l.) was slowly passed through a column (2 cm. diam.) of agar (25 g.). After 4 days this modified agar was transferred to a Buchner funnel and washed first with distilled water until free from mineral acid, and then with 0.2 M-phosphate buffer pH 7.8. In contrast to electro dialysed agar, 4 % (w/v) solutions of this washed agar in the magnesium-deficient broth together with 0.005 % (w/v)  $\text{Ca}^{++}$  set to a rigid gel.

## EXPERIMENTAL

### *Influence of magnesium on growth and cell division*

*Clostridium* and *Bacillus* spp.; Gram-positive. The abnormal, filamentous morphology of Gram-positive species of *Clostridium* and *Bacillus* grown in the magnesium-deficient medium at 37° (Table 1) revealed that magnesium was essential for normal cell division of these organisms. The formation of filaments

Table 1. *Growth of Bacilliaceae in a magnesium-deficient peptone medium*

Cultures in 'ammonia precipitated' Evans peptone broth grown for 18 hr. at 37°; magnesium concentration  $> 0.00003$  %.

| Organism   | Morphology   |
|--|--|
| <i>Cl. tertium</i>                                 | Filaments and chains   |
| <i>Cl. sporogenes</i>                              | Filaments and chains   |
| <i>B. subtilis</i> var. <i>viscosus</i> (No. 2587) | Long filaments   |
| <i>B. subtilis</i> (No. 3610)                      | Filaments, distorted filaments, chains and some normal cells           |
| <i>B. polymyxa</i> (No. 1380)                      | Filaments and shorter rods   |
| <i>B. vulgatus</i> (No. 2588)                      | Long filaments together with normal cells in chains                    |
| <i>B. mycoides</i> (No. 2602)                      | Long filaments together with normal cells in chains                    |
| <i>B. megatherium</i> (No. 2605)                   | Filaments and longer cells in chains                                   |
| <i>B. anthracis</i>                                | Long filaments and chains of normal rods extending over several fields |

was not altered by serial subculture of the bacilli in the magnesium-deficient medium, but these filamentous cells gave rise to cells of normal appearance when subcultured in a medium containing Evans peptone (2 % w/v), NaCl (0.5 %, w/v) and glucosc (0.2 %, w/v). The more abundant growth that occurred in this medium presumably indicates that magnesium plays some part in protoplasmic synthesis as well as in cell division.

In agreement with the results obtained with *Cl. welchii* (Webb, 1948a), no change in morphology occurred when filaments from 14 hr. cultures of *B. subtilis*, *B. mycoides* and *B. vulgatus* in the magnesium-deficient medium were incubated at 37° either in 0.1 % (w/v) magnesium sulphate, or in autolysed normal cultures of the organisms. On the magnesium-deficient solid medium the aerobic bacilli either failed to grow or grew with difficulty. *B. megatherium*, for example, formed scattered irregular terraced colonies with ragged or lobate margins, which were composed of long cells in chains.

In agreement with the studies of Hinshelwood (1946) on the growth of *Bact. lactis aerogenes* (*Aerobact. aerogenes*) the growth temperature had a pronounced influence on the production of filaments in the liquid medium. Thus, in cultures



of *Cl. tertium*, *B. subtilis* var. *viscosus* and *B. mycoides* incubated at 18°, the growth was poor and was composed of many normal cells together with some chains and filaments. Better growth occurred in cultures of *Cl. welchii*, *Cl. tertium* and *B. anthracis* at 25° and, in each case, the cells were in long chains. At these lower temperatures, where growth was slow, the final stationary population appeared to be sharply limited by the magnesium concentration and, in consequence, the incidence of cells of abnormal length was decreased.

The influence of magnesium concentration on the growth of the bacilli was strikingly illustrated when *Cl. welchii*, *Cl. tertium*, *B. subtilis* var. *viscosus*, *B. vulgatus* and *B. mycoides* were cultivated in complex media at 37°. As the magnesium concentration was increased from that of the 'ammonia precipitated' Evans peptone medium, the amount of growth increased visibly, while the morphological appearance of the cells changed from filaments to chains and then to isolated normal rods. On increasing the concentration further, the amount of growth progressively decreased and the normal rods were replaced by chains and finally by filaments (Fig. 1). The inhibitory effect of higher magnesium concentrations on cell division in particular is in accordance with the fact that enzymes activated by metallic ions in low concentrations are also inhibited by the same ions when the concentration exceeds a certain limit (cf. Clark, 1938).

*Lactobacillus helveticus*, *L. arabinosus* and *Kurthia zenkeri*; *Gram-positive*. Filamentous forms of *Kurthia zenkeri* (*Zopfius zenkeri*, No. 404), *Lactobacillus helveticus* and *L. arabinosus* were obtained in magnesium-deficient media. Magnesium is therefore essential for the normal cell division of these bacteria. The magnesium requirements of the lactobacilli were somewhat less than those of the Gram-positive bacilli and clostridia, since the poor growth in the magnesium-deficient medium at 37° was, especially in the case of *L. helveticus*, composed mainly of long chains, and a predominance of filaments was only obtained when the cultures were incubated at 40°. Excess magnesium (0.05–0.1 % w/v) also inhibited cell division and induced the production of filamentous forms.

*Gram-positive and Gram-negative cocci*. No abnormal morphology was observed in cultures of *Staphylococcus citreus* (B 9), *Staph. albus*, *Gaffkya tetragena* (*Micrococcus tetragenes*, No. 951), *Streptococcus pyogenes* (No. 2400), *Strep. faecalis* and three *Neisseria* species in the magnesium-deficient liquid medium. Several of these cocci were maintained by subculture for over a year in this medium without any variation in size. Furthermore, no changes in morphology occurred when the organisms were cultivated in the presence of excess magnesium. On the other hand, magnesium was essential for the growth of the Gram-positive and Gram-negative cocci, since all the strains studied grew with difficulty under the conditions of magnesium deficiency or excess, and on the solid magnesium-deficient medium only *Staph. citreus* was capable of growth.

*Gram-negative rods*. In contrast to the Gram-positive rods, better growth and normal cell-division occurred in magnesium-deficient cultures at 37° of the following strains: *Pseudomonas aeruginosa* (No. 1999), *Ps. prunicola* (No. 3870),

*Chromobacterium violaceum* (No. 2537), *Serratia marcescens* (*Chromobact. prodigiosum*) (No. 2302), *Proteus vulgaris* (No. 401), *Escherichia coli commune* (No. 86), *E. coli* var. *acidilactici* (*Bact. acidilactici*, No. 128), *Aerobacter aerogenes* (*Bact. lactis aerogenes*), *Aerobacter cloacae* (*Bact. cloacae*, No. 408) and *Alcaligenes faecalis* (*Bact. faecalis alcaligenes*, No. 415). Only occasional filamentous cells were observed during a minimum of ten subcultures. The only marked change

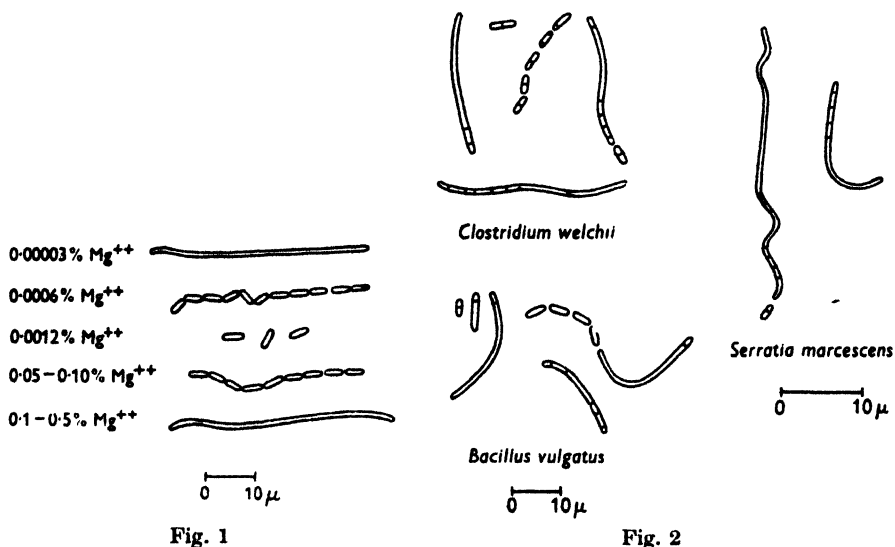


Fig. 1. Diagrammatic representation of the effect of increasing magnesium concentration on the morphology of *Clostridium welchii*, *Cl. tertium*, *Bacillus subtilis* var. *viscosus*, *B. vulgatus* and *B. mycoides*.

Fig. 2. Diagrammatic representation of the filamentous forms of *Clostridium welchii*, *Bacillus vulgatus* and *Serratia marcescens* stained to show the transverse cell walls.

was the increased polysaccharide synthesis in cultures of *Ps. prunicola* and *Aerobacter aerogenes*. The same change was observed in cultures of *Cl. welchii* in the magnesium-deficient medium (Webb, 1948*a*), and Shear & Turner (1943) found that the addition of magnesium salts to cultures of *Serratia marcescens* resulted in decreased yields of their 'haemorrhage-producing' polysaccharide.

It would appear, as with the Gram-positive organisms, that magnesium stimulated the growth of these Gram-negative organisms; the growth, as determined by opacity measurements, was invariably less in the magnesium-deficient medium than in the normal 2% peptone medium. Furthermore, Young, Begg & Pentz (1944) have shown that magnesium is essential for the normal growth of *E. coli*.

On subculture in media of greater magnesium concentration these organisms grew as long filaments. The concentration of magnesium (0.05-0.1% w/v) required to induce this change was considerably less than was the case with the Gram-positive bacteria. Indeed, higher magnesium concentrations (0.25, 0.5% w/v) often inhibited the growth of the Gram-negative species. In agreement with this, Kishimo (1927) observed that *Bact. typhosum* grew as chains and

filaments in a chemically defined medium containing 1 % magnesium sulphate while in 2 % peptone water containing magnesium the organism formed long chains. These morphological variations were not produced by other salts such as  $K_2HPO_4$ , ammonium lactate,  $CaCl_2$  and  $NaCl$ .

Division of filaments of *Ps. prunicola* and *Serratia marcescens* from cultures in the complex medium containing 0.05 % (w/v)  $Mg^{++}$  could not be induced by incubating the washed cells suspended in sterile saline at 37°.

From these results it was concluded that magnesium was equally essential for the normal cell division of the Gram-negative rods, but the critical concentrations were lower than those required by the Gram-positive bacteria. Studies of *Cl. welchii* and other Gram-positive organisms (Henry & Stacey, 1946) showed that the surface complex responsible for the Gram stain contains magnesium ribonucleate, and that this complex is absent from the Gram-negative bacteria. The Gram complex removed from *Cl. welchii* by extraction with sodium cholate contained 3.2 %  $Mg^{++}$ , whereas the residual Gram-negative cytoskeletons contained 0.1 %  $Mg^{++}$  (Henry, Stacey & Teece, 1945). From these results it is calculated that about 70 % of the total magnesium present in *Cl. welchii* (0.35 %, Webb, 1948*a*) is actually in the Gram complex. Thus if magnesium fulfils the dual role of a structural element and an enzyme activator in Gram-positive organisms, whereas in Gram-negative organisms it functions as an enzyme activator only, it is obvious that the magnesium requirements of Gram-negative bacteria will be considerably less than the requirements of Gram-positives.

The analysis (Table 2) of cells harvested from a peptone water medium of constant composition shows that, of the bacteria examined, the Gram-positive bacteria contained a higher percentage of magnesium than did the Gram-negative cells. Furthermore, when the bacterial cells were killed by heat under the conditions previously described (Webb, 1948*b*), dialysed against running tap water for 48 hr. and then slowly passed through a column of mixed ion-exchange resins (De-acidite C and ZeoKarb H.I.P. in the ratio 6:1) the Gram-positive cells retained a percentage of their magnesium, whereas with one exception, the Gram-negative cells retained none (Table 2). This result was not an artefact due to the greater magnesium concentration of Gram-positive cells, because the suspension of micrococci (T 38) was passed twice through the ion-exchange resin column before analysis. In these experiments, killed cells were used in order to avoid autolytic changes during dialysis. With certain Gram-positive bacteria, such as *Cl. welchii* and *L. plantarum*, there was a marked tendency for the cells to become Gram-negative when killed by heat unless the previously described precautions were observed. The magnesium of those cells which became Gram-negative by extraction with sodium cholate was completely removed by the ion-exchange resins (Table 2).

From these results it is concluded that magnesium is more tenaciously bound in Gram-positive than in Gram-negative bacteria. Although this bound magnesium does not correspond to the total magnesium of the Gram complex, it appears to be held by the complex, since magnesium is readily removed from the Gram-negative forms of *Cl. welchii*.

Table 2. *The removal of magnesium (and other ions) from killed Gram-positive and Gram-negative bacteria by mixed ion-exchange resins*

| Organism  | Ash (percentage of dry wt. of cells) |  | Mg <sup>++</sup> (percentage of dry wt. of cells) |  |
|---|--------------------------------------|--|---|--|
|   | Normal cells                         | Cells passed through ion-exchange resins | Normal cells                                      | Cells passed through ion-exchange resins |
| <b>Gram-positive</b>  |                                      |  |   |  |
| <i>Cl. welchii</i>  | 7.3*                                 | 2.0*                                     | 0.35*   | 0.02                                     |
| <i>Micrococcus</i> (T 38)   | 8.25                                 | 3.55                                     | 0.72  | 0.087                                    |
| <i>B. subtilis</i>  | 9.8                                  | —  | 0.70  | —  |
| <i>L. plantarum</i>   | 6.3                                  | 3.1                                      | 0.63  | 0.03                                     |
| <i>Strept. faecalis</i>   | 7.5                                  | 3.4                                      | 0.68  | 0.034                                    |
| <b>Gram-negative</b>  |                                      |  |   |  |
| <i>Chromobact. violaceum</i>  | 5.20                                 | 2.1                                      | 0.26  | Nil                                      |
| <i>Aerobact. cloacae</i>  | 6.68                                 | 3.00                                     | 0.25  | 0.019                                    |
| <i>Aerobact. aerogenes</i>  | 5.16*                                | 3.17                                     | 0.01*   | Nil                                      |
| <i>Ps. aeruginosa</i>   | 7.26                                 | 5.1                                      | 0.17  | Nil                                      |
| <i>Ps. prunicola</i>  | 3.6                                  | 1.4                                      | 0.066   | Nil                                      |
| <i>Cl. welchii</i> rendered Gram-negative by extraction with 2 % sodium cholate | —                                    | 1.8                                      | 0.1†  | Nil                                      |

\* Webb (1948a).

† Henry, Stacey &amp; Teece (1945).

*Application of specific staining methods*

Filaments of *Cl. welchii*, *Cl. tertium* and *B. polymyxa* from cultures in magnesium-deficient media, and filaments of *Ps. prunicola* and *Serratia marcescens* from cultures in media containing excess magnesium, were stained by Robinow's (1944) HCl-Giemsa method. They showed chromatinic bodies regularly spaced throughout the cytoplasm. Such cultures were 'old' (12–14 hr.) in the sense employed by Robinow, since it was considered important to distinguish filaments produced under these conditions and the shorter filamentous cells which are normally observed during the early phases of active growth of the rod-shaped bacilli (cf. Clark & Ruehl, 1922). When stained by Robinow's tannic acid-crystal violet method, transverse cell walls were observed. In some cases, these were regularly spaced along the filament. In others, only a few transverse septa were observed (Fig. 2) which divided the filaments into sections of different lengths containing varying numbers of chromatinic structures (cf. Klieneberger-Nobel, 1944).

## DISCUSSION

From the observations described here, it is concluded that the mechanism of cell division is the same in the Gram-positive and Gram-negative rod-shaped bacteria, and requires magnesium for its normal function. The differences between the magnesium requirements of the Gram-positive and Gram-negative organisms is attributed to the fact that the former also require magnesium for the formation of the Gram complex.

The stage in division which requires magnesium remains to be determined. It appears that magnesium is not essential for the division of the chromatinic bodies, for these structures are regularly distributed throughout the length of filamentous cells. If these chromatinic bodies represent true nuclei, then cell division, in the sense of the complete fission of the bacterial cell, is not a necessary consequence of nuclear division. Robinow (1945) has already shown that the onset of cell division in the rod-shaped bacteria is not related to any particular stage in the division of the chromatinic bodies. Magnesium does not appear to be primarily concerned with the formation of transverse cell-walls, since the process is only partially inhibited by magnesium deficiency or magnesium excess. The fact that the Gram-positive bacilli change from filaments to chains as the magnesium content of the medium increases towards the concentration optimal for normal cell division (Fig. 1), suggests that magnesium is involved at a stage in division between the splitting of a transverse cell-wall and the separation of the daughter cells.

The fragmentation of filamentous cells resulting either from magnesium deficiency or magnesium excess, was not observed in cultures and could not be induced artificially. In this respect, these filamentous cells differ from the shorter filaments which are observed in cultures of rod-shaped organisms during the early phase of active growth and which do subsequently divide.

The cell division of the Gram-positive and Gram-negative cocci differs from that of the rod-shaped organisms, since conditions of either magnesium deficiency or magnesium excess, although markedly inhibitory to growth, fail to produce any changes in the morphology of micrococci, sarcinae and neisseria. The streptococci examined here did not show any changes in morphology, although Bisset (1948) has observed filamentous cells in streptococcal cultures.

Presumably either many metabolic activities occur at the surface of the bacterial cell, or the material essential for growth must diffuse through the cell surface. Consequently, a limitation will be imposed upon the size of a bacterial cell by the surface/volume ( $S/V$ ) relationship, since, in general, as the size of an organism increases,  $S/V$  and, therefore, metabolism per unit volume, decreases. For a spherical coccus,  $S/V = 3/r$ , where  $r$  is the radius of the cell, and for a cylindrical 'square-ended' bacillus  $S/V = 3/d + 2/l$ , where  $d$  is the diameter and  $l$  the length of the organism. When bacilli grow in the form of filaments, as  $l$  increases  $d$  tends to decrease (cf. Webb, 1948*a*). Hence it follows that, in so far as such calculations are justified, any change in the dimensions of a cell will be of greater significance in the case of a coccus than a bacillus. Such reasoning may, in part at least, explain the fact that magnesium deficiency or magnesium excess predominantly influences the growth, and not the morphology, of the cocci.

Thanks are due to Professor M. Stacey for his interest in this work and to the Medical Research Council for a grant in aid of the expenses.

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## The Influence of Magnesium on Cell Division

### 3. The Effect of Magnesium on the Growth of Bacteria in Simple Chemically Defined Media

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**SUMMARY:** In simple chemically defined media all of 15 bacteria failed to grow in the complete absence of magnesium. The concentration of magnesium for maximum growth was dependent upon the Gram reaction of the individual species examined, the magnesium requirements of the Gram-positive organisms being some ten times greater than the requirements of the Gram-negative organisms. In contrast to the observations made in more complex media (peptone water), normal cell division occurred in chemically defined media containing suboptimal amounts of magnesium. It is suggested that magnesium is involved in the synthesis of bacterial protoplasm as well as cell division and, in simple chemically defined media, the synthetic reactions require the higher magnesium concentration.

It has been shown (Webb, 1949) that magnesium is essential for the normal cell division of the rod-shaped bacteria growing in a peptone water medium, and that it also stimulates the growth of these organisms. The Gram-positive bacteria examined required a greater magnesium concentration for normal growth and cell division than did the Gram-negative bacteria. In an attempt to interpret these results quantitatively, a study was made of the effect of magnesium on the growth of bacteria in simple chemically defined media. The bacteria studied were those capable of growth in a mixture of salts and carbon compounds, all of which could be obtained free from magnesium. The presence of traces of magnesium in some of the components of the media probably explains the conflicting results obtained by earlier workers in this field (see Knight (1936) for a review). Studies by Lodge & Hinshelwood (1939) on the growth of *Bact. lactis aerogenes* (*Aerobact. aerogenes*) in a chemically defined medium composed of pure salts and glucose, showed that small inocula would not grow in the absence of magnesium. For a given inoculum there was a limiting magnesium concentration, 1–20 parts per million (p.p.m.) according to the conditions of the experiments, below which no growth occurred and above which growth occurred normally. Moreover, the final population and, to a first approximation, the rate of growth was independent of the active amount of magnesium in excess of the limit.

In the present work, all the bacteria studied failed to grow in the complete absence of magnesium, but, in contrast to the results of Lodge & Hinshelwood (1939) maximum growth occurred at a certain optimal magnesium concentration, and this optimum varied according to the Gram-staining reaction of the organism.

#### MATERIALS AND METHODS

Water used in the preparation of media was twice distilled in a Pyrex glass still and then redistilled immediately before use. Test-tubes were cleaned in chromic-sulphuric acid mixture, rinsed in sodium bicarbonate solution and then

repeatedly washed in the distilled water. Pyrex glassware was used throughout, since media stored in soft glass vessels apparently dissolved magnesium from the glass.

The 'Analar' salts, glucose and glycerol used in the preparation of the media contained no detectable magnesium. Asparagine, used in place of aspartic acid in Koser & Rettger's (1919) medium, contained some inorganic salts which proved difficult to remove. However, neither magnesium nor manganese could be detected in the ash ( $\approx 5\%$  of the asparagine preparation). In order to minimize the precipitation which occurs in simple chemically defined media, the phosphate solutions required were sterilized separately and added to the main bulk of the medium when cold.

To avoid the transfer with the inoculum of sufficient magnesium for growth and to adapt the organism to the medium, subcultures were made from stock into the defined medium containing no magnesium and incubated for 24 hr. Serial subcultures were then made into the same medium every 24 hr. until the final subculture failed to grow. Each tube of the experimental series was then inoculated with 0.01 ml. of the penultimate culture of this series of subcultures in the Mg-deficient medium. The latter tubes were incubated at the optimum growth temperature until a stationary state was established. The amount of growth was then measured turbidimetrically by means of the Spekker adsorption photometer after the addition of 0.1 ml. of 10N-HCl. Control experiments showed that with a uniform suspension of cells (*Aerobacter aerogenes*) the readings of the instrument below 1.0 were proportional to the number of cells.

### EXPERIMENTAL

The influence of the magnesium concentration on the growth of those species examined which were able to grow in simple chemically defined media is shown in Figs. 1-7.

Owing to the thick pellicles formed by *Bacillus vulgatus* and *Mycobacterium tuberculosis*, the influence of magnesium was assessed qualitatively. *B. vulgatus* grown for 48 hr. in Koser & Rettger's (1919) medium showed the following relative amounts of pellicle at the respective Mg concentrations (in p.p.m.) indicated: -, 0; +, 5; ++, 10; +++, 20, 40 and 50; +, 100; where +++ = thick pellicle, ++ = moderate pellicle, + = thin film of growth, and - = no growth. Similarly, *M. tuberculosis*, human (a virulent laboratory strain), was grown for 8 days at 37° in Long medium (Long & Seibert, 1926), the inoculum being, per tube, a 2 mm. square piece of 8-day pellicle grown on the same medium with Mg at 0.0005% (w/v). The relative amounts of growth at the respective Mg concentrations (in p.p.m.) were: -, 0; ++, 5; +++, 50, 100 and 200; ++, 400; +, 500; where +++ = surface covered, ++ = surface  $\frac{2}{3}$  covered, + = surface  $\frac{1}{3}$  covered, and - = no growth.

All the bacteria examined failed to grow in the complete absence of magnesium. These results are in accordance with the findings of Robinson (1932) with *Ps. pyocyaneus* (*Ps. aeruginosa*) and of Lockemann (1919), Frouin & Guillaumie (1928) and Model (1929) with *M. tuberculosis* in chemically defined media.



Cultures of *Ps. prunicola* and *Serratia marcescens* in media containing sub-optimal amounts of magnesium were characterized by increased polysaccharide synthesis as indicated by the high viscosity of the cultures.

In contrast, magnesium appeared to be completely unessential for the growth of certain strains of soil actinomyces and of *Thiobacillus thiooxidans*. These organisms continued to grow in the absence of magnesium after 20 sub-cultures in chemically defined media prepared according to Conn & Conn (1941) and Waksman (1922), respectively.

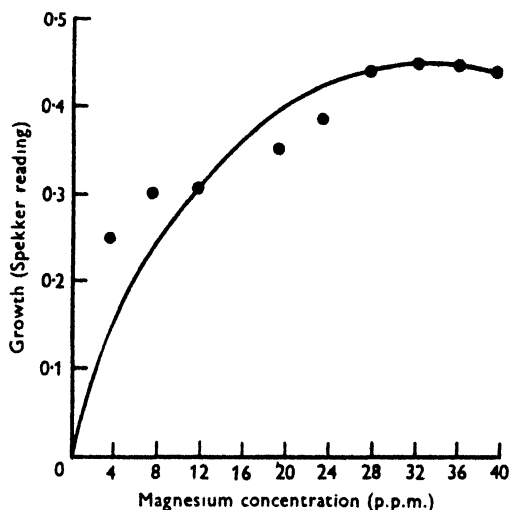


Fig. 1. Effect of magnesium on the growth of *Azotobacter chroococcum* (Burk's (1930) medium).

The curves relating magnesium concentration to the mass of growth of various organisms (Figs. 1-7) differ from those obtained by Lodge & Hinshelwood (1939) for *Aerobact. aerogenes*. Under the present experimental conditions, *Aerobact. aerogenes* attained maximal growth in two different media when the magnesium concentration was of the order of 2-3 p.p.m. Only when the initial pH value of the medium was low (pH 4.7) was the amount of growth apparently independent of the magnesium concentration (Fig. 4). However, in this case, growth was limited by the final pH of the medium, since all cultures covering the range of magnesium concentration had a final constant pH value (pH 3.46-3.53). Furthermore, when the medium was initially adjusted to pH 3.4, *Aerobact. aerogenes* failed to grow, irrespective of the magnesium concentration.

Certain of the bacteria studied (e.g. *Serratia marcescens*, *Aerobact. aerogenes*) grew in media in which the only inorganic cations were K and Mg (together with ammonia as nitrogen source). Similarly, Katznelson (1947) has shown that K, Mg and P are the only inorganic elements necessary for the growth of *B. polymyxa* in a defined medium. The presence in the Analar salts of other functionally active metallic ions such as, for example, Fe, in concentrations sufficient for growth cannot, however, be excluded (cf. Waring & Werkman, 1948).

In the present experiments neither Gram-positive nor Gram-negative

bacteria grew as filaments in the chemically defined media containing sub-optimal amounts of magnesium. Only in cultures of certain Gram-negative bacteria (e.g. *Ps. prunicola*, *Aerobact. aerogenes*) in media containing the higher magnesium concentrations used (8–10 p.p.m.) were filamentous cells observed.

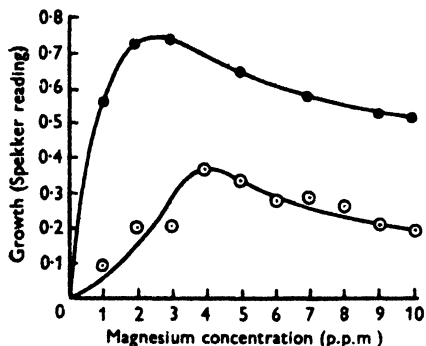


Fig. 2

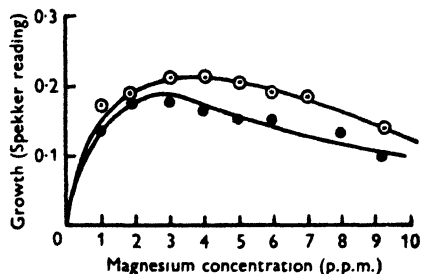


Fig. 3

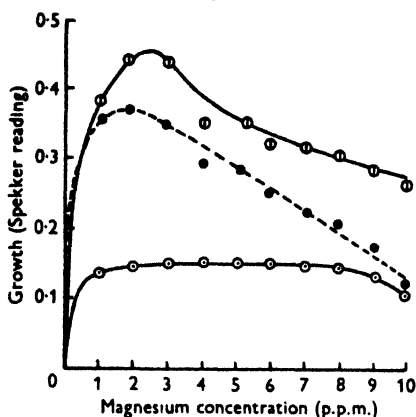


Fig. 4

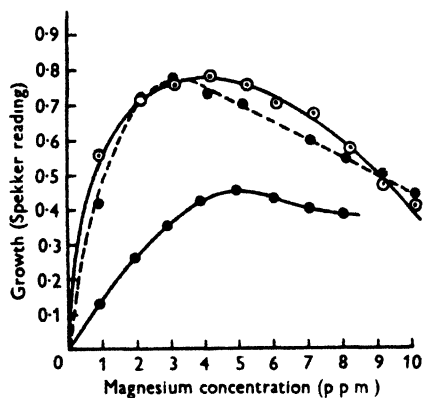


Fig. 5

Fig. 2. Effect of magnesium on the growth of *Pseudomonas aeruginosa* (●—●) and *Ps. prunicola* (○—○) (Erikson's (1945) medium).

Fig. 3. Effect of magnesium on the growth of *Alcaligenes faecalis* (●—●) and *Escherichia coli-commune* (○—○) (Koser & Rettger's (1919) medium).

Fig. 4. Effect of magnesium on the growth of *Aerobacter aerogenes*. ○—○ medium of Lodge & Hinshelwood (1939). pH 4.7. ○—● medium of Lodge & Hinshelwood (1939). pH 6.5. ●---● medium of Koser & Rettger (1919). pH 6.8.

Fig. 5. Effect of magnesium on the growth of *Aerobacter cloacae* (●—●), *Esch. coli* var. *acidilactici* (●---●) and *Chromobact. violaceum* (○—○) in Koser & Rettger's (1919) medium.

On increasing further the magnesium concentration the incidence of filaments was increased. Thus cultures of *Ps. prunicola* in Erikson's (1945) medium containing 0.5% (w/v) Mg grew as tangled filaments and microscopic examination failed to reveal any single short cells. In contrast, filaments were never observed with the Gram-positive species examined in cultures containing

magnesium in excess of the optimal concentration for growth. Such concentrations (e.g. 100 p.p.m.) often more or less completely inhibited the growth of the organisms (Fig. 7).

### DISCUSSION

Figs. 2-5 show that the Gram-negative bacteria studied reach maximum growth when the magnesium concentration is of the order of 2-4 p.p.m. In contrast, maximum growth of the Gram-positive bacilli examined occurred at a magnesium concentration of 20-40 p.p.m. (Fig. 7). At concentrations of

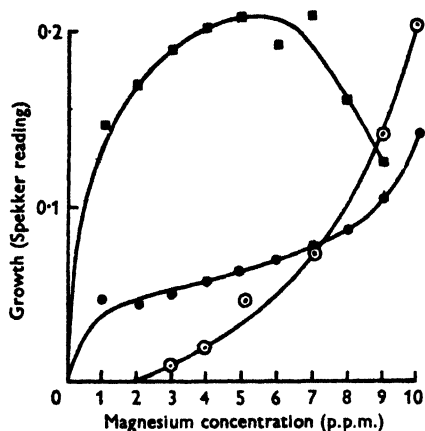


Fig. 6

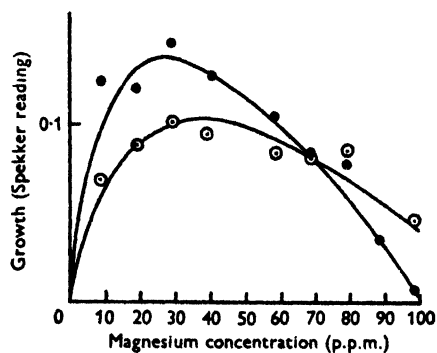


Fig. 7

Fig. 6. Effect of magnesium on the growth of *Serratia marcescens* (■—■), medium of Shear, Turner, Perrault & Shovelton (1943), *Bacillus subtilis* var. *viscosus* (●—●) and *B. polymyxa* (○—○) (Koser & Rettger's (1919) medium).

Fig. 7. Effect of magnesium on the growth of *B. mycoides* (○—○) and *B. subtilis* (●—●) in Koser & Rettger's (1919) medium.

magnesium which were optimal for the Gram-negative bacteria the growth of the Gram-positive bacteria was only a fraction of the maximum value (Fig. 6). Although the relatively small number of species studied does not justify generalization, it would appear from these and previous results (Webb, 1949) that Gram-positive bacilli require a much greater concentration of magnesium for optimal growth than do Gram-negative bacteria. This difference can be attributed to the fact that the Gram-positive organisms incorporate magnesium into the structure of the Gram complex.

The fact that growth in simple chemically defined media is a function of the magnesium concentration and that no growth occurs at zero concentration suggests that magnesium is essential for the synthesis of bacterial protoplasm. Magnesium is also essential for the normal activity of the cell-dividing mechanism (Webb, 1949). Hence in the development of a normal bacterial population, magnesium takes part in (1) the synthesis of substances intermediate in composition between the foodstuff provided and the bacterial protoplasm, and (2) cell division. Furthermore, it is conceivable that in a complex medium which contains preformed components of protoplasm, such

as amino-acids and other essential metabolites, a deficiency of magnesium would predominantly affect the process of cell division and not that of synthesis of protoplasm. On the other hand, in a simple chemically defined medium, on which an organism which was a good synthesizer could grow, magnesium would be essential both for synthesis of cell substance and for cell division. Under these conditions of growth, synthesis precedes cell division and, presumably, requires the higher magnesium concentration. That is, at concentrations of magnesium below the optimum for growth, normal cell division occurs throughout the restricted population that the medium is able to support. Only at relatively high and partially inhibitory concentrations of magnesium is the balance between growth and cell division altered to such an extent that filamentous cells are formed, i.e. cell division is impeded by the high magnesium concentration.

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## *Bacillus polymyxa* and its Bacteriophages

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**SUMMARY:** Thirty strains of *Bacillus polymyxa* were isolated from soil, vegetation and water, and nine strains were received from other sources. At the time of isolation the new strains were of a white or sporogenous colony type, but rapidly produced asporogenous and rough variants. Four bacteriophages were isolated from a variant of a soil strain and from soil and sewage. These differed in host-strain specificity, particle size, heat resistance, citrate sensitivity and serological reactions.

Following the report by Ainsworth, Brown & Brownlee (1947) of an antibiotic—'Aerosporin'—produced by *Bacillus aerosporus* (Greer), work was undertaken on certain aspects of the bacteriology of this organism. *B. aerosporus* is considered by Porter, McCleskey & Levine (1937) to be identical with *B. polymyxa*, and on morphological, cultural, biochemical and serological grounds, we agree with this opinion.

Samples of soil, vegetation and water were obtained from widely separated localities in Great Britain, and these were cultivated by the methods described by Ledingham, Adams & Stanier (1945). From these samples 30 strains of *B. polymyxa* were isolated. In addition, 4 strains were received from Dr Ainsworth's group, and 5 strains from other workers.

The main characters of this organism have been reported by Greer (1928), Porter *et al.* (1937) and Smith, Gordon & Clark (1946). In the present paper it is proposed to record only certain morphological and colonial characters of the organism and the isolation and examination of four strains of bacteriophage active against it.

### *Colonial variation in B. polymyxa*

Ledingham *et al.* (1945) illustrated a bewildering variety of colony types of *B. polymyxa*. The description of the 'typical' colony by Smith *et al.* (1946) is undoubtedly that of the rough type and is typical of the two strains which we obtained from the National Collection of Type Cultures, No. 1380, and the American Type Culture Collection, No. 8523, whereas the colony described by Smith *et al.* as rough is the white or sporogenous parent colony.

Since the method of isolation employed included the pasteurization of soil samples before culture, the majority of strains were isolated as sporogenous colonies. Subsequent study has shown that these represent the parent colony type, since variants are thrown from them which have never reverted to the parent type.

Presumptive identification of colonies on the plating medium of Ledingham *et al.* (1945) was made on morphological grounds and the development of typical spores. Likely colonies were subcultivated in nutrient broth, and only those developing obvious ropiness were further examined. Final identification was

made on biochemical grounds, and by bacteriophage and serological tests. The serological methods will be the subject of a later communication.

With freshly isolated strains, the typical colony developed in cultures on nutrient agar after 24 hr. incubation at 28° is smooth, flat or low convex, circular, slightly brownish and about 1 mm. in diameter. After 48 hr. it is 2–3 mm. in diameter, low convex, white and opaque with an entire edge. The 24 hr. culture consists of vegetative bacilli about  $3 \times 1\mu$ , but at 48 hr. a large proportion of the bacilli are forming central or subterminal spores and have become spindle-shaped. After 3 days or longer, the culture consists mainly of oval spores, surrounded by a rim of stainable cytoplasm, which subsequently disappears.

When broth cultures of the white colonies are left at room temperature for a few days and then plated, it is often found that in addition to the white (sporogenous) colonies there are other colonies, which after 2 or 3 days' incubation remain brown, though of the same size as the white colonies (Pl. 1, fig. 1). These brown colonies consist of vegetative bacilli, many of which have undergone partial autolysis, and only occasionally can spores be found. These colonies do not revert to the white type.

When broth cultures of white or of brown colonies are left for 7 days or longer at room temperature, typical rough colonies also appear (Pl. 1, fig. 2). These breed true and do not revert; when plated on soft or rather moist agar, they tend to swarm and to form a thin, grey sheet of growth.

A fourth or mucoid type of colony was also found in a few cultures of brown variants kept for many weeks in the cold room, but this type could not be isolated with any regularity. These colonies are much larger than the brown colonies, often 4 mm. in diameter; after 2 or 3 days' incubation they are markedly umblicated (Pl. 1, fig. 3) and are more glairy and sticky than the brown colonies. In stained films the appearance closely resembles that of the brown colony, showing vegetative bacilli, many partially autolysed bacilli and few spores; but individual bacilli tend to be smaller than those of the typical brown colony.

Sporogenous strains that have become largely replaced by brown or rough variants may be 'rejuvenated' either by heating broth cultures at 55° or by prolonged storage of agar slope cultures in the cold room, even when white colonies cannot be demonstrated by direct plating.

It should be mentioned that different strains vary widely in the speed with which they produce variants. Some strains remain stable for many weeks at room temperature; others throw brown or rough variants which largely replace the parent colony type in a week or two at room temperature. Storage of cultures in the cold-room almost abolishes this variation. The amount of mucinous substance formed by different strains varies, and this modifies the colonial appearance to some extent. Rough variants produce less mucinous substance, both on plates and in nutrient broth, and much more is produced in media containing added carbohydrate. The exact nature of the mucinous substance has not been determined.

BACTERIOPHAGES FOR *B. POLYMYXA**Sources and methods of isolation*

Four different strains of bacteriophage active against *B. polymyxa* were isolated.

Phage A was isolated (August, 1947) from culture-tanks in which *B. polymyxa* CN 1984\* was being grown for polymyxin production. This phage was subsequently propagated on a brown colony variant isolated from *B. polymyxa* CN 1417, of which CN 1984 is a mucoid variant. CN 1417 was isolated from soil by Ainsworth *et al.* (1947).

Phages B and C were both isolated from a pool of soil samples during a search for a phage which would attack CN 2002, a strain of *B. polymyxa* resistant to phage A. Nine samples of Sussex soils and waters were pooled after cultures for *B. polymyxa* had been prepared from them individually. Five of the soil samples subsequently yielded strains of *B. polymyxa*. The pool of soil samples was added to 100 ml. glucose phosphate peptone water (methyl red, Voges-Proskauer medium). The mixture was inoculated with *B. polymyxa* CN 2002, incubated for 2 days at 28° and the culture filtered through paper and then through a Doulton porcelain filter-candle. The bacteria-free filtrate was propagated daily on *B. polymyxa* CN 2002 for one week, and the final culture filtered as before. Serial decimal dilutions spotted on an agar plate freshly inoculated with *B. polymyxa* CN 2002 showed the presence of phage in high titre. This phage did not produce confluent lysis in the true sense, since a thin film of phage-resistant organisms was always found covering the area of lysis.

The above crude preparation was found to attack all the strains of *B. polymyxa* available at that time. The appearance of the plaques varied on different strains of *B. polymyxa*, and grey haloes were often formed in the growth around the 'phaged' areas. A pure Phage B was isolated from a single plaque. Some strains of *B. polymyxa* proved to be resistant to the action of this phage.

Phage C was isolated (March, 1948) from the same crude phage by picking a plaque from a plate spread with *B. polymyxa* CN 2366, a strain resistant to phage B. This phage C, which was further purified, attacked almost all strains of *B. polymyxa* tested, and gave complete lysis of the host strain CN 2366 on an agar plate culture.

Phage D was isolated (July, 1948) from sewage taken from the main drain of these laboratories. The sewage was diluted with an equal volume of glucose + phosphate + peptone water and divided into nine parts. Each of these samples was inoculated with a different strain of *B. polymyxa* and was incubated at 28° overnight. After paper and filter-candle filtrations, the bacteria-free filtrates were inoculated into cultures of the respective host strains. After 24 hr. incubation five cultures showed good bacterial growth, but four remained clear and phages were isolated from these. The plaque appearances produced by the phages isolated from these four cultures corresponded with one another

\* 'CN' numbers are the culture numbers in the bacterial collection of the Wellcome Research Laboratories, Beckenham.



on every strain of *B. polymyxa* on which they were tested. They were therefore assumed to be identical, and the purified phage propagated on *B. polymyxa* CN 2161 was labelled phage D.

#### Activity of *B. polymyxa* phages

The four phages differ in their host range among the strains of *B. polymyxa* (Pl. 2, figs. 1-4). Phage C attacked all but one (CN 1576) of the strains against which it was tested. Phages A, B and D differ in the strains which they can attack. Table 1 shows the grouping of the *B. polymyxa* strains on the basis of their phage sensitivity. This grouping so far cannot be correlated with any other property of *B. polymyxa*.

Table 1. *Distribution table of sensitivity of strains of B. polymyxa to four types of bacteriophage*

| Strains of <i>B. polymyxa</i> (CN numbers)           | Sensitivity to phages |   |   |   |
|--|-----------------------|---|---|---|
|  | A                     | B | C | D |
| 1417, 1418, 1971, 2222, 2225, 2227, 2228, 2368, 2490 | +                     | + | + | + |
| 2224   | +                     | + | + | - |
| 2161, 2229, 2369, 2370, 2492                         | +                     | - | + | + |
| 1419, 1951, 1966, 2002, 2136, 2159, 2180, 2184, 2185 | -                     | + | + | + |
| 2366   | +                     | - | + | - |
| 2185, 2168, 2164, 2163, 2191                         | -                     | + | + | - |
| 2181, 2182, 2401                                     | -                     | - | + | + |
| 2162, 2179, 2186, 2220, 2230, 2491                   | -                     | - | + | - |

These *B. polymyxa* phages have been tested against seventeen other members of the genus *Bacillus*. A single strain of each of the following species, classified according to Smith *et al.*, was tested.

Group 1. *B. megatherium*, *B. cereus*, *B. cereus* var. *mycoides*, *B. subtilis*, *B. subtilis* var. *aterrimus*, *B. subtilis* var. *niger*, *B. pumilus*, *B. coagulans*, *B. firmus*.

Group 2. *B. macerans*, *B. circulans*, *B. laterosporus*.

Group 3. *B. sphaericus*, *B. sphaericus* var. *rotans*, *B. sphaericus* var. *fusiformis*.

None of these cultures was lysed by any of the four *B. polymyxa* phages.

Phage A is the most virulent of the four phages. It causes complete lysis on a plate and in liquid culture. Secondary growth is very rare and usually does not appear in liquid medium even on prolonged incubation. With all four phages the appearance of the plaques varies considerably with the strain of bacillus under test, and with the dryness and thickness of the agar. Phage A gives clear plaques, 0.5-2.0 mm. in diameter. The edges may be entire or with sharp indentations. Haloes up to 1 cm. in diameter develop on keeping the plate at room temperature.

Phage B never gives complete lysis on a plate and resistant organisms always develop in liquid culture. On a plate culture of a susceptible strain, the area of lysis is always covered by a thin film of growth (Pl. 2, fig. 5). Individual plaques vary between 0.5 and 2.0 mm. in diameter, and the edges are usually ill defined. Haloes are sometimes present, and are visible on plates when removed from the incubator. The bacteria which grow in the presence of phage B are

resistant to it. Single colonies obtained by plating a broth culture of the growth scraped from the area of 'confluent lysis' were resistant to phage B. Filtrates of broth cultures grown from such colonies may contain a phage that resembles phage B in its behaviour and is inhibited by an antiserum prepared against phage B. Phage has not been found in broth filtrates of normal strains.

Although phage C completely lyses the host strain CN 2866 on solid medium, secondary growth may occur in liquid medium with some strains, but this is not constant. The plaques formed by the C phage on first isolation were very small, often invisible but for a large halo. However, after repeated culturing on strain *B. polymyxa* CN 2866, the plaques increased in size, although their appearance remains very variable. We have evidence suggesting that propagation of phage C on different host strains results in changes in specificity in a manner analogous to that of Craigie's type II typhoid phage (Craigie & Yen, 1988).

Some strains of *B. polymyxa* are completely resistant to phage D on solid medium, others are fully susceptible, or secondary growth may develop in the areas of lysis. In a liquid medium, growth always appears within 48 hr. even if the phage-treated culture remains clear after 24 hr. incubation. The plaques vary between 0.5 and 4 mm. in diameter. Well-defined haloes are sometimes present, and with a few strains there is a well-marked white rim of increased bacterial growth around the area of lysis and within the halo.

#### *Inter-strain inhibition in B. polymyxa*

Early in work with phage A it was found that broth-filtrates of one strain of *B. polymyxa* might inhibit the growth of another, and even produce an area of complete inhibition of growth, resembling phage lysis, when a drop of bacteria-free filtrate or of broth-culture was spotted on a plate spread with the second strain. At first this was thought to be due to phage, and that one strain was lysogenic for another. On dilution, however, the area of inhibition became fainter, and at dilutions greater than 1 in 100 it usually disappeared. True phage plaques were never demonstrated (Pl. 1, fig. 4). The antibiotic effect of strains of *B. polymyxa* upon one another has been reported by Katznelson (1944), who also encountered a bacteriophage for this species.

Strains of *B. polymyxa* differ, among other things, in the type of polymyxin produced: five polymyxins are now recognized (Brownlee & Jones, 1948). It was thought that there might be some correlation between the inter-strain antibiotic effect and the polymyxin types involved. Accordingly all available strains of *B. polymyxa* were tested according to the method of Fisk (1942). A remarkable series of results was thus obtained. The patches of growth of the test cultures might merge into the growth of the underlying strain or they might be distinct from it but without inhibitory effect. Inhibition varying from a greyish ring to marked inhibition resembling phage lysis might be present (Pl. 1, fig. 5). A few strains inhibited themselves under these conditions, but only to a minor extent.

The strains could be grouped on the basis of the number of strains which they inhibited, and on their resistance to inhibition, but no useful classification

resulted. Moreover, the type of polymyxin produced bore no relation to the antibiotic activity of any particular strain. The inhibitory material was produced more readily on solid medium than in broth. This inhibitory effect of one strain of *B. polymyxa* upon another does not appear to be related to the substance produced by *B. polymyxa* which inhibits *Staph. aureus* (Stansly & Schlosser, 1947). A strain which inhibits few other *B. polymyxa* strains will lyse *Staph. aureus* just as readily as one which is active against most other strains of its own species. Numerous attempts to isolate a phage from actively inhibitory strains have failed. Artificial lysogenic strains exist in the form of cultures of phage B-resistant organisms, but we have not encountered a naturally lysogenic culture.

#### *Differential properties of the bacteriophages*

In addition to the different lytic activities of the four phages, other properties were examined. Phage B is distinguished by its serological specificity, its large particle size, and its greater heat resistance relative to the other three phages. Phage D is citrate-resistant. Phages A and C are closely related and cannot be clearly separated by any of the tests employed, but three serial passages of each phage on the host strain for the other have not changed the strain specificity of either. All four phages are inhibited by the methylene-blue test and the urea test described by Burnet (1933*b*).

**Particle size.** By the use of collodion membranes prepared by the methods of Elford (1931) we made an approximate determination of the average particle-size of the four phages. The phage preparations, consisting of filtered broth-cultures, were diluted with equal volumes of fresh nutrient broth, and filtered at pH  $7.5 \pm 0.1$ . All preparations were filtered through a membrane of average pore diameter  $0.64 \mu$  before being subjected to a filtration test through membranes of smaller pore diameter. No attempt was made to determine the end-point of filtration for any phage. The following results show the range between two membranes defining the phage size:

Phage A = 33–60  $m\mu$ : probably nearer the upper limit.

Phage B = 60–113  $m\mu$ : probably nearer the upper limit.

Phage C = 33–88  $m\mu$ : probably nearer the lower limit.

Phage D = 21–83  $m\mu$ : probably nearer the lower limit.

Phage B is thus distinguished from the other three phages by its markedly larger diameter.

**Heat resistance.** All four phages are remarkable for their heat lability. The results of heating suspensions of these phages in nutrient broth at pH 7.2 are shown in Table 2. Phage B is again distinct, in that considerable activity remains after heating to  $50^\circ$ .

**Growth rate.** The final phage concentration of all four phages when propagated on their respective host strains in nutrient broth and incubated at  $28^\circ$  overnight is of the order of  $10^8$  particles/ml. In 6 hr. phages A, C and D will have almost reached this concentration, but phage B multiplies much less rapidly. The rate of phage multiplication is much diminished at  $37^\circ$  as compared with that at  $28^\circ$ , with all four phages.

Counts of the number of phage particles liberated from one host cell have been made, by the method of Burnet (1929). With all four phages, the burst-count appears to be of the order of  $2^7$ – $2^8$  (128–256). The burst-time with phage A is about 150 min., with phage B somewhat over 360 min., with phage C and

Table 2. *Heat resistance of B. polymyxa bacteriophages*

Samples of phage suspensions were diluted 1 in 10 in nutrient broth at a final pH of 7.2. Five ml. volumes in  $6 \times \frac{1}{2}$  in. test-tubes were heated for 20 min. in water-baths at the required temperatures.

Counts given represent numbers of phage particles in 1/2000 ml. of the undiluted phage preparations. The figures for 45° were obtained from a separate experiment.

|               | Unheated control | Phage suspension heated at |        |       |       |
|---------------|------------------|----------------------------|--------|-------|-------|
|               |                  | 45°                        | 50°    | 55°   | 60°   |
| Phage A       |                  |                            |        |       |       |
| Count         | 120,000          | —                          | 52     | 4     | 0     |
| Survivors (%) | 100              | 24                         | 0.043  | 0.003 | 0     |
| Phage B       |                  |                            |        |       |       |
| Count         | 70,000           | —                          | 24,000 | 12    | 0     |
| Survivors (%) | 100              | 100                        | 34     | 0.017 | 0     |
| Phage C       |                  |                            |        |       |       |
| Count         | 46,000           | —                          | 0      | 0     | 0     |
| Survivors (%) | 100              | 6                          | 0      | 0     | 0     |
| Phage D       |                  |                            |        |       |       |
| Count         | 215,000          | —                          | 100    | 40    | 2     |
| Survivors (%) | 100              | 100                        | 0.047  | 0.019 | 0.001 |

phage D about 200 min. Confirmation of the first two figures has been obtained by another method. Plaque counts were made from phage preparations incubated with the host culture over a longer period (up to 8 hr.). The counts were plotted on a logarithmic scale and the time required for the count of phage particles to double at the maximum growth rate was read off. This gives a doubling division time or mean generation time of 18 min. for phage A and of 45 min. for phage B. If the burst count is taken as  $2^8$  for each phage, the mean generation times deduced from the first experiment would be  $18\frac{1}{2}$  min. for phage A, somewhat over 45 min. for phage B and about 25 min. for phages C and D. In non-aerated nutrient broth the mean generation time for *B. polymyxa* at 28° is a little over 1 hr.

Thus, phages A, C and D multiply at much the same rate and have similar burst-times; phage B multiplies much more slowly and differs markedly from the other three.

*Serological relationships.* A method similar to that of Burnet (1933*a*) was used. Antisera were prepared by the injection of rabbits with filtered broth-culture lysates. Serial dilutions of the antisera were mixed with equal volumes of the phage preparation under test, so diluted that 0.1 ml. of the mixture gave about 100 phage plaques. The mixtures were incubated at 37° for 2 hr. and 0.1 ml. volumes then spread on an agar plate freshly inoculated with the host strain. Titres are recorded as the final dilution of the serum which gives a count amounting to 20 % of the control count. The results of a typical experiment are shown in Table 3.

**Citrate sensitivity.** This was determined by the method of Burnet (1933*b*), modified to give a roughly quantitative result. Serial tenfold dilutions of the phage preparation were dropped in 0.02 ml. quantities on nutrient agar plates to which had been added 0, 0.1, 0.25, 0.5, 1.0 and 1.5 % sodium citrate, and

Table 8. *Serological relationships of B. polymyxa phages*

| Antiserum<br>to phage | Inhibitory titre of antiserum with phage |        |        |       |
|-----------------------|--|--------|--------|-------|
|                       | A  | B      | C      | D     |
| A                     | 19,200                                   | 0      | 19,200 | 4,800 |
| B                     | 0  | 38,400 | 0      | 0     |
| C                     | 2,400                                    | 0      | 2,400  | 600   |
| D                     | 4,800                                    | 0      | 2,400  | 9,600 |

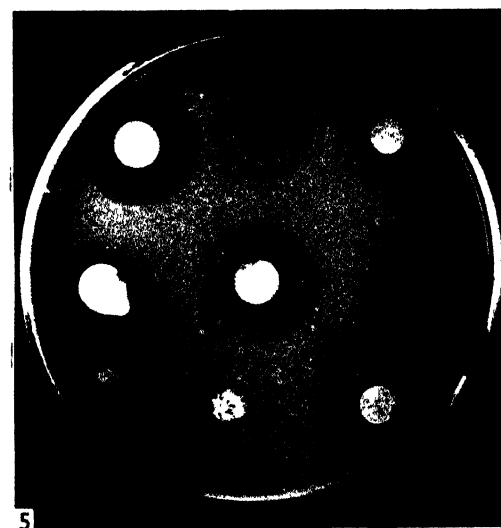
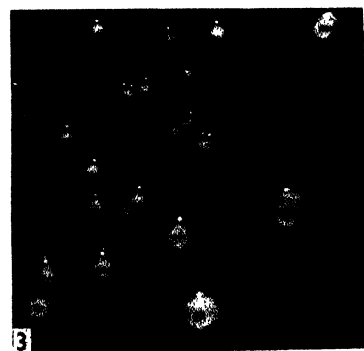
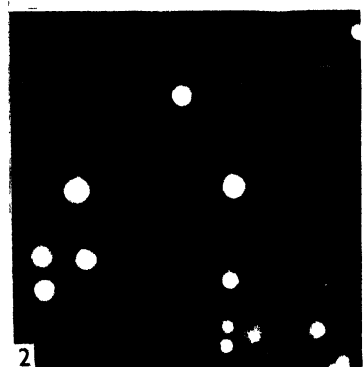
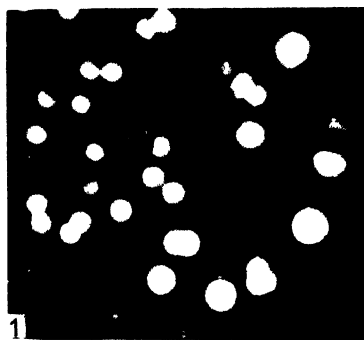
'0' indicates no inhibition by the serum at a dilution of 1 in 300.

which had been freshly inoculated with the appropriate strain of *B. polymyxa*. It was found that phages A and C were decreased to 10 % of their initial activity by 0.25 % citrate and almost inactivated by 0.5 %; phage B was decreased to 10 % of its initial activity by 0.5 % citrate and almost inactivated by 1 %; phage D was unaffected by 1.5 % citrate even though growth of the host strains was diminished by this concentration.

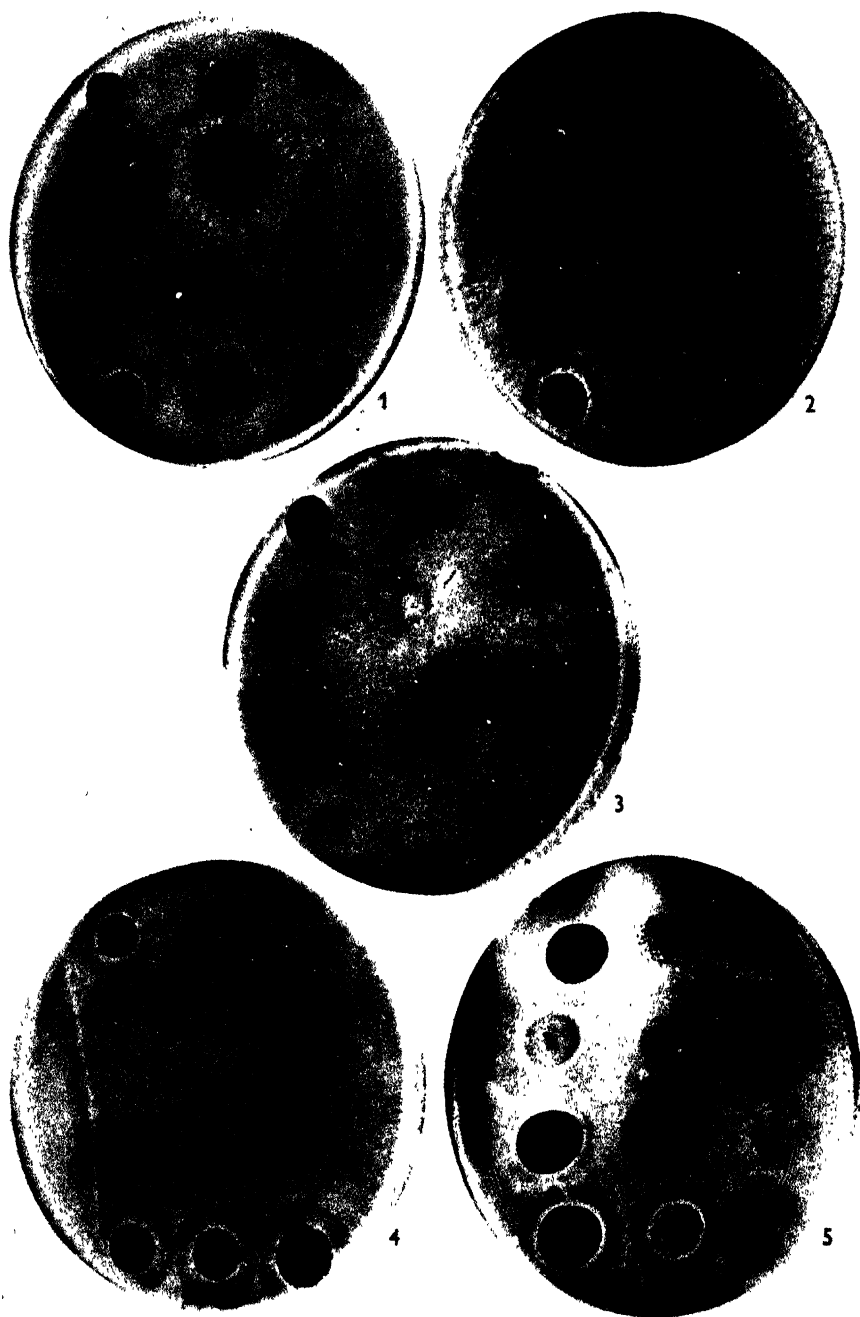
We are indebted to Dr C. L. Oakley for the collodion membranes and for advice on the technique of the phage filtrations. We wish to thank Mr E. A. Jones and Mr E. E. Hitchcock for the photographs, and Mr M. S. Beer and Mrs A. N. Cook for valuable technical assistance.

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Figs. 1-5



Figs. 1-5

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## EXPLANATION OF PLATES

### PLATE 1

Figs. 1, 2 and 3. Magnification  $\times 2$ .

- Fig. 1. *B. polymyxa* CN 1417 showing white and brown colonies.
- Fig. 2. *B. polymyxa* CN 1966 showing white and rough colonies.
- Fig. 3. *B. polymyxa* CN 1417, mucoid variant.
- Fig. 4. Inter-strain inhibition. Filtrate of broth culture of *B. polymyxa* CN 1417 spotted on *B. polymyxa* CN 2002 in serial decimal dilutions, anticlockwise.
- Fig. 5. Inter-strain inhibition. Plate spread with strain *B. polymyxa* CN 1966. Loopsful of broth cultures of nine other strains spotted on the plate. Incubated 2 days at 28°.

### PLATE 2

All figures show plate cultures of host strains of *B. polymyxa* with phages A, B, C and D arranged vertically; at dilutions of 1/10, 1/10<sup>3</sup> and 1/10<sup>5</sup> from left to right.

- Fig. 1. CN 1417, original host strain for phage A. Shows reaction with phages A, B, C and D.
- Fig. 2. CN 2002, original host strain for phage B. Shows reaction with phages B, C and D; also antibiotic inhibitory effect with phage A preparation.
- Fig. 3. CN 2366, original host strain for phage C. Shows reaction with phages A and C.
- Fig. 4. CN 2161, original host strain for phage D. Shows reaction with phages A, C and D.
- Fig. 5. CN 2222. Shows reaction with phages A, B, C and D, with film of resistant organisms across areas of lysis with phage B. (Owing to the mucinous nature of broth cultures of *B. polymyxa* it is difficult to prevent the growth of colonies in what should be areas of confluent lysis, as with phages A, C and D in this example.)

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## Origin, Development and Significance of L-forms in Bacterial Cultures

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**SUMMARY:** Several Gram-negative bacteria produce pleuropneumonia-like (L-) forms, some under ordinary cultural conditions, others only when exposed to abnormal ones. The formation of these bodies starts with the production of small nuclear elements surrounded with a thin cytoplasmic layer; these elements fuse with neighbouring elements and the L-body is complete. Whereas some L-strains reproduce themselves indefinitely, others revert to the bacterial form. Thus the bacterial and the L-forms are now regarded as two different generations of the same organism and my symbiosis theory is abandoned.

Symbiosis was my explanation of the association between pleuro-pneumonia-like organisms that breed true (L-forms) and *Streptobacillus moniliformis* (Klieneberger, 1935, 1942). In contrast, Dienes and others believe that the L-form is either a variant or a phase in the life cycle of the *Streptobacillus*. Dienes's view (1947, 1948) that the L-forms arise from a simple swelling of the bacterium, that the large L-forms are a reproductive phase and the small L-forms are bacteria-like is too simple; my opinion is, and always has been, that the bacterial and the L-forms differ fundamentally in cytology, colony form, metabolism (Partridge & Klieneberger, 1941), and in pathogenicity. A typical young bacterium (which I designate an A-form) has a wall of considerable thickness (see Fig. 1, Knaysi & Baker, 1947) which gives rigidity to the cell. Inside the wall is a cytoplasmic layer, which becomes septate during division, enclosing the transversely arranged chromatinic structures called 'chromosomes' by Robinow (1944, 1945). At division a ring-like band forms on the inner side of the cell-wall and gradually cuts the cell in two but without obvious constriction of the outer part of the cell-wall. In contrast, the L-forms have only a thin cell boundary which may be no more than a fine layer of condensed cytoplasm; the cells are plastic and because they can assume any shape I have described them as amorphous. They divide by segmentation into two or more parts (see Figs. 8 and 9, Smith, Hillier & Mudd, 1948), the size and shape of these elements being determined by the physical conditions of the environment (Klieneberger & Smiles, 1942). Chromatinic material may be present in big clumps or in particles too small for the resolving power of the light microscope; it may assume different shapes, including filaments (mycelium- or network-like), but it never shows the characteristic bacillary arrangement.

With these differences in mind it was clear that the origin and development of the A- and L-forms needed to be studied by the newer cytological methods developed by Robinow and by Boivin. This has been done with certain Gram-negative bacteria, some of which produce L-forms under ordinary cultural conditions, others only in special conditions that are often adverse to the growth of the organism.

### Methods

Young liquid cultures were inoculated on the solid medium and small squares of the agar cut out; these were placed inoculated side downwards on sterile coverslips, and incubated in a moist chamber. After incubation the agar square was removed and the wet coverslip film fixed in osmic acid vapour. Fixed preparations either without further treatment, or after treatment with warm N-HCl, or with a solution of ribonuclease, were stained with a weak Giemsa solution. Occasionally Bouin's fixative was applied through the agar; this was followed by tannic acid as a mordant and by dilute crystal violet to stain. (For further details of the methods applied see Klieneberger-Nobel, 1945; Robinow, 1944, 1945; Boivin, 1948; Boivin, Tulasne, Vendrely & Minck, 1947 *d*; Tulasne & Vendrely, 1944).

## OBSERVATIONS

### *Formation of L-growth under ordinary conditions of culture*

*Fusiformis necrophorus* (*Bacteroides funduliformis*). Strain 132, isolated by Dienes, was subcultured once a week for two years in Brewer's medium enriched with horse serum. The inoculum was large (3 or 4 drops) and was incubated at 36° for only 7 hr.; between subcultures, cultures were kept in the cold. Before each experiment the strain was subcultured three times a day on two successive days, when it consisted entirely of A-forms. When inoculated on to plates of 'special medium' (boiled blood agar made from ox-heart infusion peptone broth enriched with horse serum) these bacilli multiplied without lag phase and after 2-3 hr. anaerobic incubation the chromatinic structures ('chromosomes') were arranged more or less transversely in the cells (Pl. 1, fig. 1). With longer incubation these structures divided into smaller ones and many appeared as granules. The cytoplasm now stained more delicately than before and seemed to be poor in ribonucleic acid, for very little treatment was needed to show its chromatinic structures (Pl. 1, fig. 2); cellular outlines could hardly be demonstrated at this stage (3-4 hr.). A little later single nuclear granules and rows of granules embedded in delicately staining cytoplasm were seen almost exclusively; each granule, with its faint surrounding cytoplasm, seemed to represent one small unit (the 'primary cell unit') which tended to coalesce with neighbouring units (Pl. 1, figs. 3*a* and 3*b*) after 4-5 hr. incubation. In further development the nuclear granules grew into fine filaments (Pl. 1, figs. 3*a* and 3*b*) which fused on contact with similar filaments or other nuclear granules (Pl. 1, fig. 6) to form a chromatinic network (Pl. 1, figs. 7-9). The nuclear matter might now contract towards the centre and leave free a uniform, lightly stained, cytoplasmic edge to form a large disk-like body (Pl. 1, fig. 10), which, because of its shape, size and structure, must be regarded as an L-body. Neighbouring primary cell units fused in twos, threes, or more up to fairly big complexes to produce a large L-body. Pl. 1, figs. 4 and 5 show fields in which only a few units have joined together. Two primary cell units belonging to different chains of primary cell units might

join to form a small body of the two united elements with a filamentous appendage on either side; this formation resembled a bacillus with a bulbous swelling. The size and shape of the L-bodies largely depended on the number and arrangement of the primary cell units which joined together and explains the bizarre shapes the L-body often assumes. The young bodies, small or large, were sometimes extremely thin in substance but they soon built up their chromatinic and cytoplasmic material. The arrangement of the chromatinic material in the fully developed elements was not constant; sometimes it appeared as a chromatinic network, as finely dispersed granules, and sometimes it had a coarse appearance. With further incubation the L-elements often increased in size and divided, by segmentation and constriction, into two or more parts at the same time.

In liquid and semi-solid media subdivision appeared to be more frequent and the single bodies were smaller than the giant flat bodies produced on the surface of solid media. After 5–7 hr. incubation almost all the growth of strain 132 often developed into L-forms (Pl. 2, fig. 11). Later the bodies showed condensation of the chromatinic material and the formation of finger-like protuberances into which the concentrated, darkly staining, filamentous nuclear material migrated. These protuberances broke away and appeared as young, well-defined and well-stained bacilli containing one or more conspicuous nuclear structures or 'chromosomes' (Pl. 2, fig. 12). After two days' incubation the culture was almost completely transformed into the A-form.

However, L-growth may remain stable, producing no A-forms. I have isolated from strain 132 two pure L-lines which were kept for a year and underwent 100 passages without reverting (Klieneberger-Nobel, 1947). Therefore we must conclude that while the L-form ordinarily produces the A-form it may—under conditions not yet defined—persist in the L-form for an indefinite time. On the other hand, the L-form is produced by the A-form at a special stage of its development by the union of small elements, called the primary cell units.

*Streptobacillus moniliformis*. This species has been studied extensively in the past and I have isolated the L-form (then called L<sub>1</sub>) in pure culture from several strains from different sources; one strain has undergone 650 passages in the course of 15 years and bred true to the L-type. The strain used for the present work, mouse 2, was kept in the laboratory for several years and for the last two years was maintained by the methods used for *F. necrophorus*, which caused it to grow almost entirely in the A-form; scanty L-growth was found only on the second day of incubation. On the first day growth was mainly composed of small, slender bacilli; however, at the edges of stained coverglass microcultures, groups of disintegrating bacilli could be found occasionally. Later fine nuclear structures appeared in similar places and their configurations suggested that they might be on the point of joining together. Later still groups of L-bodies were found at the edges of the preparations but a substantial amount of L-growth was never detected. Thus by regular transfers for two years, a culture which originally produced L-forms in abundance was so changed that in the first 24 hr. it produced only A-forms and a few L-forms only during the second

day. *S. moniliformis* is the smallest of these organisms studied here; consequently the transformation from the A- to the L-form was more difficult to follow than in any of the other organisms. But I have no doubt that the transformation is basically the same as in *F. necrophorus*.

*Bacterium coli*. Strain 204 was obtained from Dr F. Kaufmann; its pleomorphism was noticed at first examination. After a few hours' growth on trypsin digest agar its bacillary forms disintegrated into very small elements which soon combined with each other (Pl. 2, fig. 13 and Pl. 4, fig. 37), and a little later the culture consisted almost exclusively of L-bodies (Pl. 2, figs. 13 and 14). After a period of L-growth the bodies reproduced the A-form; at this stage the nuclear material was very dense, stained darkly (Pl. 4, fig. 35) and had divided into chromosome-like filaments. In Pl. 4, fig. 35, the angular body below the large body seems to have been breaking up into bacillary forms; it is surrounded by a number of bacilli, apparently newly formed, having the darkly stained nuclear structure typical of young bacilli. Strain 204 was more penicillin-resistant than any other strain of the *Bact. coli* and salmonella groups tested; it grew without inhibition on nutrient agar containing 800 units penicillin/ml. All L-strains, as well as pleuropneumonia-like organisms, were found to be penicillin resistant. Morphologically strain 204 was the same on penicillin and penicillin-free media.

#### *Formation of L-growth under the influence of various stimuli*

*Change in temperature.* A motile and well-flagellated strain of a *Proteus* sp. was studied. A needleful of dried culture was placed in the centre of a well-dried plate and the organisms were allowed to grow at room temperature until a fairly large swarming edge had been produced; the plates were then kept in the cold room (0.5°) for three days (see Ørskov, 1947). Plates were then incubated at 36° for several hours and impression preparations were taken from the swarming edge every quarter- or half-hour. When taken from the cold the swarming edge showed a number of normal bacteria; others were transparent and contained a row of small chromatinic granules; free granules were also found between disintegrating bacterial filaments. It is supposed that the granules, together with a cytoplasmic envelope not always demonstrable by the methods employed, represent the primary cell units because after a short incubation they form delicate ramifications. It seems that by means of these ramifications neighbouring granules join to form larger mycelium-like chromatinic formations (Pl. 3, fig. 26). These chromatinic networks and the surrounding cytoplasm build up and take stains more easily so that fully developed L-bodies were usually detected after 1½–2 hr. incubation (Pl. 3, fig. 27). The number of L-bodies produced by this method varied; apparently it depended on the stage in which the bacteria at the swarming edge were caught when transferred to the cold.

To prove this, undried plates were evenly inoculated with 1–2 drops of a young broth culture, incubated for 1, 2, 3, 4 or more hours and placed in the cold for three days. They were then reincubated and impression preparations taken during incubation. L-forms did not develop on plates given only 1 hr.

of preliminary incubation, a few appeared in the 2-hr. plate, and the largest number were produced on the plate given a preliminary incubation of 8 hr. With longer incubation the number of L-forms decreased. The production of L-forms and the rapidity of their appearance was stimulated by very rich media; thus meat infusion peptone agar was superior to trypsin digest agar. When the L-forms were once established they multiplied more slowly than the A-forms, so that they were soon outnumbered. After 5-6 hr. the L-bodies underwent a change; the nuclear material condensed and the body formed finger-like processes into which the nuclear material migrated. The protuberances broke away and the bodies produced bacillary elements in much the same way as the L-bodies of *F. necrophorus*. After 12 hr. incubation L-forms were no longer detected.

*Effect of sodium chloride.* It has been known for a long time that 'large bodies' develop under the influence of salts. A strain of *Pasteurella pestis*, N.C.T.C. 144, was studied on various media with and without added NaCl. During the first 5-6 hr. the organisms developed similarly on all the media. Media were usually inoculated at night with a young broth-culture, kept at room temperature until morning and then transferred to the incubator. After a few hours' incubation on NaCl (8 %) agar a change occurred; the cells stained less deeply and their nuclear structure showed up on staining without previous treatment. Sometimes the cytoplasm was unstainable and the outline of the cells was lost; Pl. 2, fig. 16, shows a chain of normal cells flanked on either side by naked chromatinic structures. Many tortuous filaments showing numerous loops and bends might be found at the same time (Pl. 2, figs. 15, 17 and 18). Soon afterwards the cytoplasm of these loops and bends seemed to coalesce, the enclosed nuclear structures or granules formed fine ramifications by which they joined together and produced round and oval L-bodies (Pl. 2, figs. 15 and 17). Sometimes parallel bacterial filaments joined lengthwise, probably by combination of opposite primary cell units; a much wider filament was thus produced, with chromatinic matter in the network formation or condensation peculiar to L-growth (Pl. 2, fig. 19). By the union of a large number of primary cell units composite bodies of very peculiar appearance were produced (Pl. 2, fig. 20).

*Effect of lithium chloride.* The action of lithium chloride on Gram-negative organisms has often been studied and most workers agree that the large bodies developing under its influence produce normal bacteria when transferred to media not containing lithium. Yet how bacteria form the large bodies has not been examined in great detail. Accordingly *Bacterium coli-mutabile*, N.C.T.C. 2495 was grown in nutrient agar containing lithium chloride in concentrations of 0.05 and 0.1 %. The lithium salt produced a conspicuous effect, particularly at the higher concentration. During the first few hours the nuclear structures of the bacilli were arranged in the 'chromosome' pattern and cell divisions took place. Later the cells had swollen slightly and the cell outline was not clearly defined; the cytoplasm was very thin and the nuclear content had been transformed into small structures or granules which filled the interior of the cells. Subsequently the cells broke up into primary cell units (Pl. 3, fig. 21).

Disintegration was not always complete, for some of the units stayed together in the arrangement they occupied in the mother cell. The free chromatinic granules produced fine ramifications by which neighbouring units combined (Pl. 3, fig. 24) and the nuclear network increased and acquired a greater affinity for the stain; the cytoplasm also became visible (Pl. 3, fig. 22). As the culture aged, round and oval bodies differentiated out of the complexes of young L-matter; these bodies were flat disks on the surface of the solid medium. With aging, their dispersed nuclear matter condensed, leaving free a delicately staining cytoplasmic edge. At this stage (Pl. 3, fig. 23) the bodies sometimes showed a delicate outline but a real cell-wall was never seen. In the low concentrations of lithium used the L-bodies invariably produced the A-forms without transfer to new medium; after about 12 hr. the large bodies had divided up into smaller ones (Pl. 3, fig. 25) containing condensed nuclear matter; in some, finger-like protuberances into which nuclear matter had migrated could be seen. The protuberances became detached, their nuclear material divided into 'chromosomes' and the new bacteria were born.

*Effect of penicillin.* Penicillin often produces aberrant forms in bacteria (Gardner, 1940; Boivin *et al.* 1947; Braun & Yalim, 1948). Four organisms, *Bact. coli-mutabile*, N.C.T.C. 2495; a *Proteus* sp., and *Salmonella paratyphi-B* 801 and 802, were examined and behaved alike; the development of one, *S. paratyphi-B* 802 will be described. Penicillin concentrations were chosen so that noticeable inhibition was avoided; 3, 7.5, and 15 units/ml. were used for the paratyphoid strains and up to 200 units/ml. for the more resistant organisms. At first the bacilli developed in the usual way and revealed the ordinary set of 'chromosomes'; after 4-6 hr. incubation they stained less well and consisted of long, slender, and often involved filaments; cultures on penicillin-free media consisted of rods. The nuclear material broke up and became less distinct and the cells disintegrated into granular material (Pl. 4, fig. 28). Often transparent bacterial filaments filled with a row of granules were seen and the granules were gradually set free. I believe that these granules represent the primary cell units which, by union with their own kind, produce the L-forms. After about 5 hr. incubation the small units were numerous (Pl. 4, fig. 31). One half to one hour later these granules developed ramifications which seemed to fuse with their neighbours; at the same time they increased in size and stainability (Pl. 4, fig. 30). Occasionally tortuous filamentous masses were found at the same time (Pl. 4, figs. 29 and 32). The primary cell-units developing in these involved filaments are presumably closely packed and in joining up, transform the whole clump of filaments into one large L-body; the various shapes of the resulting L-bodies are seen in Pl. 4, figs. 29a, b, c and 32; other aspects of the fusion are shown in Pl. 4, figs. 34, 37 and 39. The irregular outlines of newly formed bodies (Pl. 4, figs. 39) can be understood only when it is realized that they have been produced by the combination of many small units. The period of fusion is followed by a period of building of nuclear and cytoplasmic substance, so that many well-coloured bodies, both large and small, are found between the bacterial filaments (Pl. 4, fig. 38). This is followed by a period of multiplication of the L-bodies.

After 18 hr. many L-bodies have produced bacteria (Pl. 4, fig. 88); the bacteria are recognized as young organisms by their well-stained and typically arranged 'chromosomes' (Pl. 4, fig. 86). In the left-hand corner of Pl. 4, fig. 86, a large L-body shows the deeply stained chromatinic material which seems to have broken up into short filaments which may represent the 'chromosomes' of the developing bacteria.

## DISCUSSION

Dienes believes that the L-form represents a reproductive phase of bacteria. Tatum & Lederberg's work (1947) is suggestive of the existence of a sexual phase in bacteria. To prove that the L-cycle is connected with such a phase it would be necessary to show that the L-form is the product of a union of two bacterial elements, and that bacteria can be produced from the L-form. The only recently recorded evidence of union of bacterial elements is a single observation by Smith (1944) with a strain of *Fusiformis necrophorus*. Several workers (Dienes, 1948, 1948a; Dienes & Smith, 1944; Smith, Mudd & Hillier 1948) have shown that bacteria may arise from L-forms and these observations have been confirmed by the work reported in this paper.

The first stage in the development of the L-form is the formation of very small cells (primary cell units) consisting of a chromatinic granule and a thin surrounding cytoplasm, poor in ribonucleic acid. These units fuse in a characteristic way: first the cytoplasm coalesces and then the nuclear granules produce ramifications which combine with neighbouring chromatinic structures. This union might be regarded as sexual, but it can involve any number of units from two upwards. Union is followed by an increase in both nuclear and cytoplasmic material and the L-body is then formed. Reproduction also is characteristic of the L-body and differs from that of the A-form; there is segmentation and constriction into two or more elements. To call small rod-like L-elements bacilli, as Dienes and Smith *et al.* have done, is misleading; structure, not size and shape, is the crux of the problem; the structure is that of an L-form; size and shape are dependent on the physical conditions of the environment. However the evidence produced from living material (Dienes & Smith, 1944), by the electron microscope (Smith *et al.* 1948), and, in this paper, from stained preparations, leaves no doubt that true bacteria (A-forms) can develop from L-forms. Thus the circle is complete: A-forms produce L-forms by the fusion of special elements, and the L-forms can produce A-forms.

It seems as if almost all Gram-negative bacteria can go through the L-cycle, some under ordinary cultural conditions, others only when affected by abnormal conditions; these include temperature changes, appropriate concentrations of sodium chloride, or lithium chloride, and penicillin in sublethal concentrations. In 1942 I expressed the opinion that the large 'salt forms' differed from typical L-forms such as those of *Streptobacillus moniliformis*, but my recent observations showed that the 'bodies' were always produced in the same way and therefore must be regarded as L-forms. Finally, the evidence is now so conclusive that A- and L-forms are two distinct phases of the same organism that my symbiont theory is untenable.

I should like to thank Mr L. J. Hale and Miss Jacqueline Schoppig for valuable technical assistance and Dr S. T. Cowan for his help with the manuscript.

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## EXPLANATION OF PLATES

(Magnification  $\times 3000$ )

## PLATE 1

- Fig. 1. *Fusiformis necrophorus* '132', 3 hr. 36°, osmic acid, ribonuclease, Giemsa (boiled blood medium plus serum was used for '132' throughout).
- Fig. 2. *F. necrophorus*, '132', 5½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Figs. 3a and b. *F. necrophorus*, '132', 5½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Figs. 4 and 5. *F. necrophorus*, '132', 7 hr. 36°, osmic acid, ribonuclease, Giemsa.
- Fig. 6. *F. necrophorus*, '132', 5½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 7. *F. necrophorus*, 5½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Figs. 8 and 9. *F. necrophorus*, '132', 7½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 10. *F. necrophorus*, '132', 7½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.

## PLATE 2

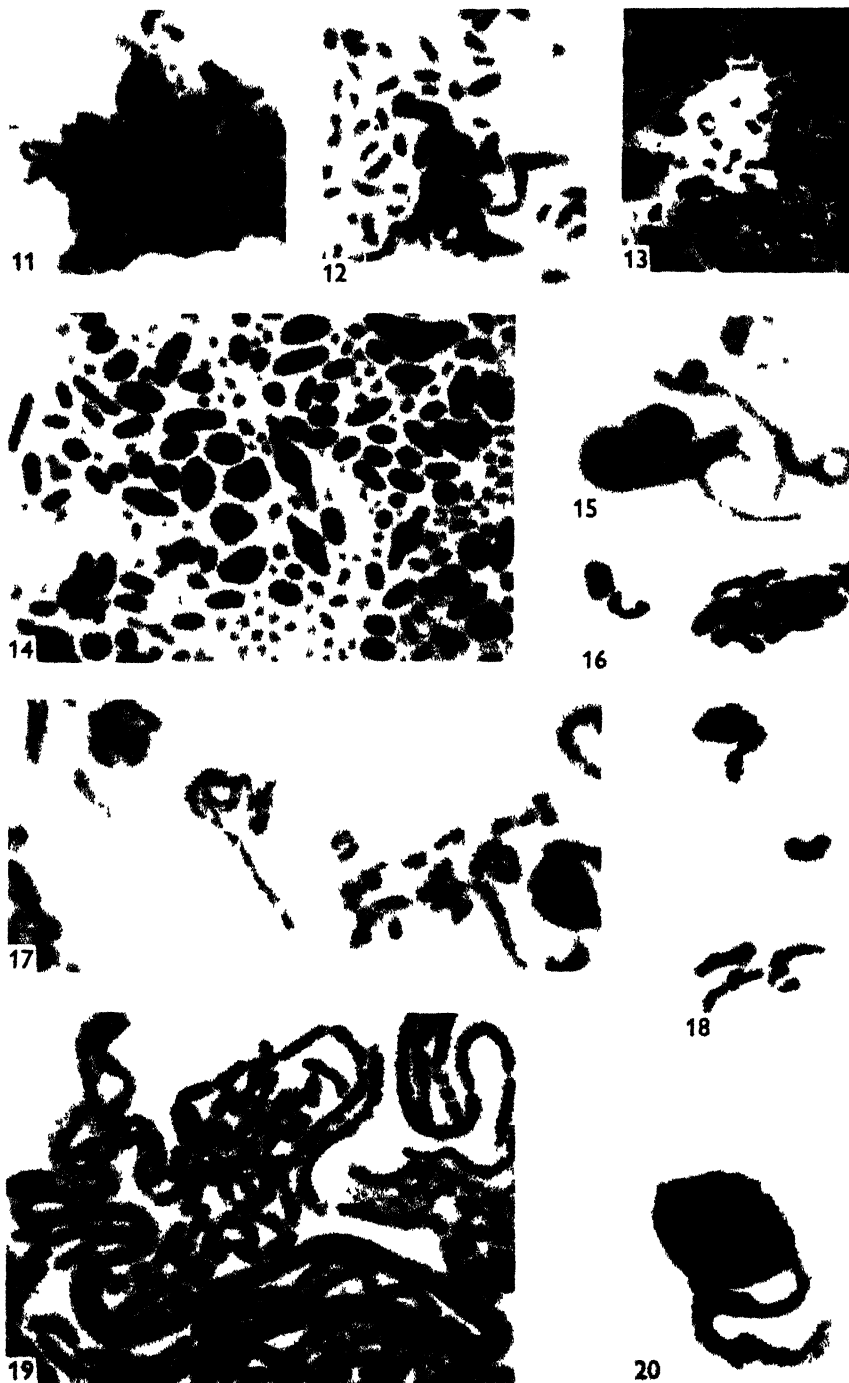
- Fig. 11. *F. necrophorus*, '132', 7 hr. 36°, osmic acid, ribonuclease, Giemsa.
- Fig. 12. *F. necrophorus*, '132', 24 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 13. *Bact. coli*, '204', meat infusion peptone agar, 5 hr. 36°, osmic acid, Giemsa.
- Fig. 14. *Bact. coli*, '204', meat infusion peptone agar, 5 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 15. *Pasteurella pestis*, '144', boiled blood medium, 2% sodium chloride, overnight room temperature, 5 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 16. *Past. pestis*, ordinary nutrient agar, 6 hr. 36°, edge of micro-culture, osmic acid, ribonuclease, Giemsa.
- Fig. 17. *Past. pestis*, '144', boiled blood medium, 2% sodium chloride, overnight room temperature, 5 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 18. *Past. pestis*, '144', ordinary nutrient agar, 6 hr. 36°, 1% sodium-chloride, osmic acid, ribonuclease, Giemsa.
- Fig. 19. *Past. pestis*, '144', ordinary nutrient agar, 1% sodium-chloride, overnight room temperature, 1½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 20. *Past. pestis*, '144', ordinary nutrient agar, overnight room temperature, 2 hr. 36°, 1.5% sodium-chloride, osmic acid, ribonuclease, Giemsa.

## PLATE 3

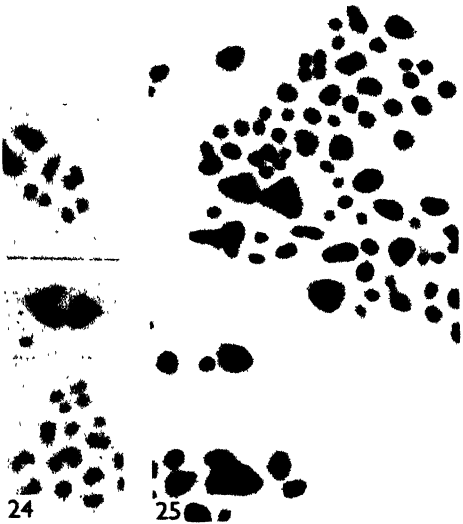
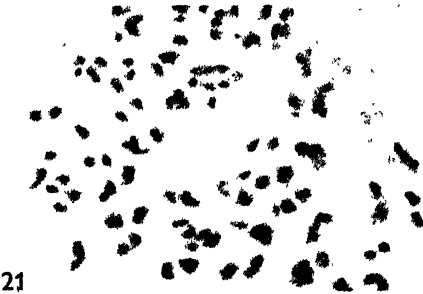
- Fig. 21. *Bact. coli mutabile*, '2495', nutrient agar plus 0.1% lithium chloride, 6½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 22. *Bact. coli mutabile*, '2495', nutrient agar plus 0.1% lithium chloride, 9 hr. 36°, osmic acid, hydrochloric acid, Giemsa.



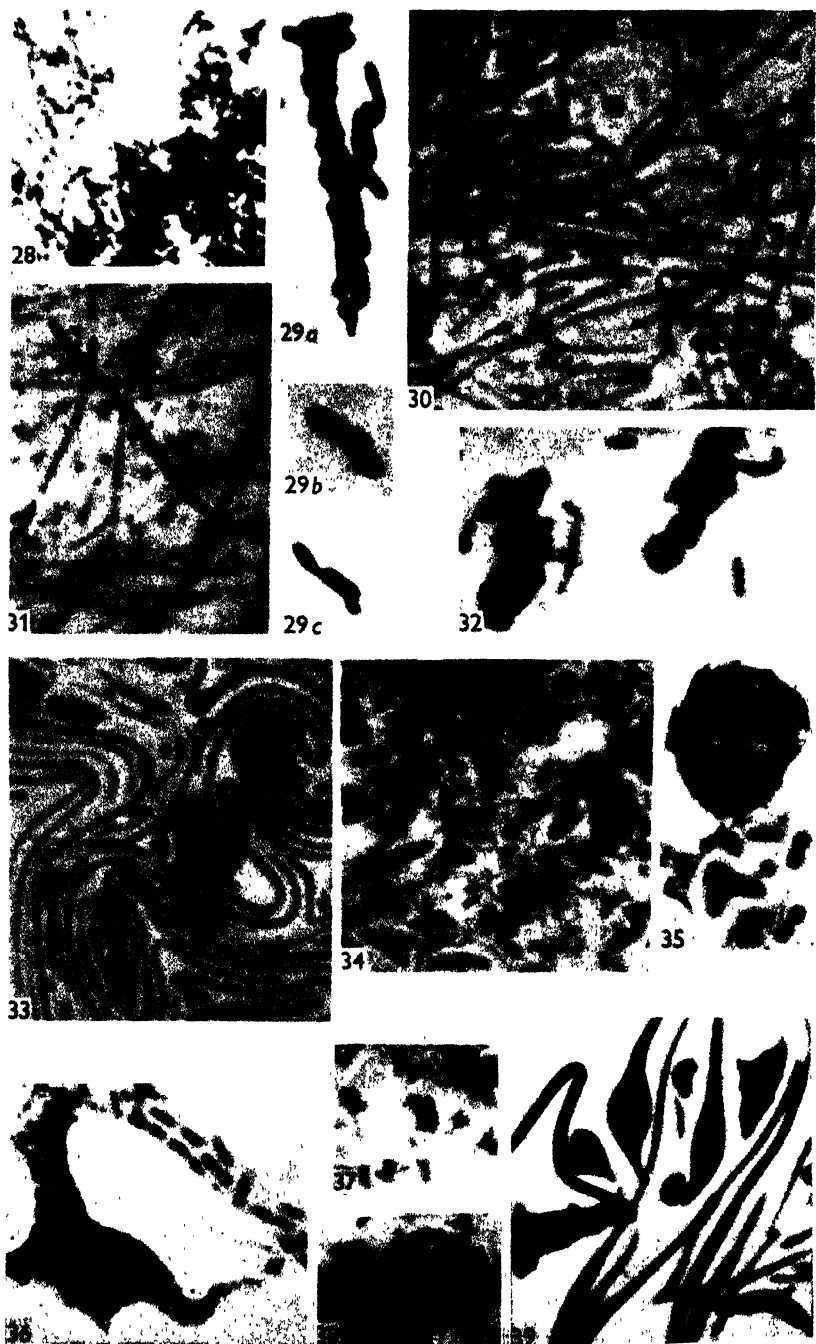
Figs. 1-10



Figs 11-20



Figs. 21-27



Figs. 28-38

- Fig. 23. *Bact. coli mutabile* '2495', nutrient agar plus 0.1 % lithium chloride, 8 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 24. *Bact. coli mutabile* '2495', nutrient agar plus 0.2 % lithium chloride, overnight room temperature, 6 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 25. *Bact. coli mutabile* '2495', nutrient agar plus 0.1 % lithium chloride, 9 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 26. *Proteus* sp., meat infusion peptone agar with horse serum, 3 hr. incubation, then cold room for 3 days, reincubated 1½ hr., osmic acid, hydrochloric acid, Giemsa.
- Fig. 27. *Proteus* sp., swarming edge, 4 days cold room, reincubated for 2 hr., osmic acid, hydrochloric acid, Giemsa.

## PLATE 4

- Fig. 28. *Salmonella paratyphi-B*, '802', meat infusion peptone agar, 20 units of penicillin/ml. medium, 4 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 29a, b and c. *Bact. coli mutabile* '2495', nutrient agar, 40 units of penicillin/ml. medium, 2 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 30. *Salmonella paratyphi-B* '802', meat infusion peptone agar, 20 units penicillin/ml. medium, 4½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 31. *S. paratyphi-B*, '802', meat infusion peptone agar, 20 units penicillin/ml. medium, 5 hr. at 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 32. *S. paratyphi-B*, '801', meat infusion peptone agar, 40 units penicillin/ml. medium, 3½ hr. at 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 33. *S. paratyphi-B*, '802', meat infusion peptone agar, 20 units penicillin/ml. medium, 6 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 34. *Proteus* sp., boiled blood medium, 800 units penicillin/ml. medium, osmic acid, hydrochloric acid, Giemsa.
- Fig. 35. *Bact. coli*, '204', boiled blood agar, 400 units penicillin/ml. medium, during the day at 36° and then room temperature overnight, osmic acid, hydrochloric acid, Giemsa.
- Fig. 36. *Proteus* sp., nutrient agar, 400 units penicillin/ml. medium, 18 hr. 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 37. *Bact. coli*, '204', nutrient agar, 800 units penicillin/ml. medium, 2-3 hr. 36°, osmic acid, Giemsa.
- Fig. 38. *Proteus* sp., nutrient agar, 400 units penicillin/ml. medium, 18 hr. at 36°, osmic acid, hydrochloric acid, Giemsa.
- Fig. 39. *Proteus* sp., nutrient agar, 400 units penicillin/ml. medium, 3½ hr. 36°, osmic acid, hydrochloric acid, Giemsa.

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# International Bacteriological Code of Nomenclature

EDITED BY R. E. BUCHANAN, R. ST JOHN-BROOKS  
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The following Bacteriological Code of Nomenclature was developed by the Judicial Commission, approved and recommended by the Nomenclature Committee and adopted unanimously at the concluding Plenary Session of the International Association of Microbiologists at the fourth International Congress held in Copenhagen, Denmark, in July, 1947.

In 1948 it was published in the *Journal of Bacteriology*, 55, 287, but in view of its importance, it is reprinted here by permission of the Editor and Proprietors of that Journal.

## CHAPTER I

### GENERAL CONSIDERATIONS

1. The progress of bacteriology can be furthered by a precise system of nomenclature which is properly integrated with the systems used by botanists and zoologists and accepted by the majority of bacteriologists in all countries. Bacteriological nomenclature considers bacteria, related organisms, and the viruses. Botanical and zoological codes provide for nomenclature of certain groups such as the yeasts and fungi, protozoa and algae. These are of such significance in the microbiological laboratory that provision is necessary in the bacteriological code for the consideration of special nomenclatural problems in these groups and for co-ordination of findings with zoologists and botanists.

2. The precepts on which this system of bacteriological nomenclature is based are divided into *principles*, *rules*, and *recommendations*.

The *principles* (Chapter 2) form the basis of the rules and recommendations.

The *rules* (Chapter 3) are designed (1) to make effective the principles given in Chapter 2, (2) to put the nomenclature of the past into order, and (3) to provide for that of the future. They are always retroactive; names or forms of nomenclature contrary to a rule (*illegitimate names or forms*) cannot be maintained.

The *recommendations* deal with subsidiary points, their object being to bring about greater uniformity and clearness, especially in future nomenclature; names or forms contrary to a recommendation cannot on that account be rejected, but they are not examples to be followed.

3. *Provisions* for emendation of rules, for special exceptions to rules, and for their interpretation in doubtful cases have been made through the establishment of a Nomenclature Committee for the International Association of Microbiologists and its Judicial Commission (Chapter 4).

## CHAPTER 2

## GENERAL PRINCIPLES

**Principle 1.** The essential points in nomenclature are (1) to aim at fixity of names; (2) to avoid or to reject the use of forms and names which may cause error or ambiguity or throw science into confusion. Next in importance is the avoidance of all useless creation of names. Other considerations, such as absolute grammatical correctness, regularity or euphony of names, more or less prevailing custom, regard for persons, etc., notwithstanding their undeniable importance, are relatively accessory.

(See *Rules 23, 24, 25, 26, 27; Recommendations 27a-i.*)

**Principle 2.** In the absence of a relevant rule, or where the consequences of rules are doubtful, established custom must be followed. In doubtful cases a résumé in which all pertinent facts are outlined should be submitted to the Judicial Commission for an Opinion.

(See *Recommendation 9c; Provision 4.*)

**Principle 3.** Bacteriological nomenclature and botanical nomenclature are interdependent in the sense that the name of a bacterial group is to be rejected if it is a later homonym of the name of any plant group. Likewise nomenclature of bacteria and protozoa are interdependent; the name of a bacterial group is to be rejected if it is a later homonym of the name of a protozoan group. Bacteriological nomenclature is independent of zoological nomenclature (protozoology excepted); the name of a bacterial group is not to be rejected simply because it is identical with the name of a group in the animal kingdom.

(See *Rule 24 (4).*)

**Principle 4.** Scientific names of all groups are usually taken from Latin or Greek. When taken from any language other than Latin, or formed in an arbitrary manner, they are treated as if they were Latin. Latin terminations should be used so far as possible for new names.

(See *Rules 1-8, 27, 28; Recommendations 5a, 6a, 6b, 6c, 8a, 27a-i.*)

**Principle 5.** Nomenclature deals with (1) the *terms* which denote the rank of taxonomic groups (such as species, genus, family, order); (2) the *names* which are applied to the individual groups (such as *Bacillus subtilis*, *Streptococcus*, *Spirillaceae*, *Spirochaetales*).

(See *Principle 7; Rules 1-8; Recommendations 6a-c, 8a, 24a.*)

**Principle 6.** The rules and recommendations of bacteriological nomenclature apply to all bacteria, recent and fossil, with certain distinctly specified exceptions.

(See *General Considerations 1; Principle 9; Provisions 2-4.*)

**Principle 7.** The terms which denote the rank of taxonomic groups are defined as follows:

(a) Every individual belongs to a species, every species to a genus, every genus to a family, every family to an order, every order to a class, every class to a division. In some families the rank tribe may be distinguished.

(See *Principle 5; Rules 1-8; Recommendations 5a, 6a-c.*)



(b) In many species, subspecies or varieties are distinguished; in some cases subdivisions of a species such as strains, groups, serotypes, variants, phases, and others may be recognized. In some genera, subgenera may be distinguished.

(See *Rules* 6, 7; *Recommendations* 6a-c, 8a.)

(c) If a greater number of intermediate categories (ranks) are required, the terms for these subdivisions are made by adding the prefix 'sub-' to the terms denoting the ranks. Thus subfamily denotes a rank between a family and a tribe, subtribe a rank between a tribe and a genus, etc. The classification of subordinate categories (ranks) may thus be carried for the bacteria in the following order.

- |                                      |                                      |
|--------------------------------------|--------------------------------------|
| 1. Division ( <i>Divisio</i> )       | 9. Tribe ( <i>Tribus</i> )           |
| 2. Subdivision ( <i>Subdivisio</i> ) | 10. Subtribe ( <i>Subtribus</i> )    |
| 3. Class ( <i>Classis</i> )          | 11. Genus ( <i>Genus</i> )           |
| 4. Subclass ( <i>Subclassis</i> )    | 12. Subgenus ( <i>Subgenus</i> )     |
| 5. Order ( <i>Ordo</i> )             | 13. Species ( <i>Species</i> )       |
| 6. Suborder ( <i>Subordo</i> )       | 14. Subspecies ( <i>Subspecies</i> ) |
| 7. Family ( <i>Familia</i> )         | 15. Variety ( <i>Varietas</i> )      |
| 8. Subfamily ( <i>Subfamilia</i> )   | 16. Individual ( <i>Individuum</i> ) |

(d) The definition of each of these categories (ranks) varies, up to a certain point, according to individual opinion and the state of the science; but their relative order, sanctioned by custom, must not be altered. No classification is admissible which contains such alteration.

**Principle 8.** The primary purpose of giving a name to a taxonomic group is not to indicate the characters or the history of the group, but to supply a means of referring to it.

(See *Rule* 28.)

**Principle 9.** Each group with a given circumscription, position, and rank can bear only one valid name, the earliest that is in accordance with the Rules of Nomenclature. Provisions may be made for certain exceptions.

(See *Principle* 6; *Rules* 24-26; *Provisions* 2, 3, 4.)

**Note.** In subgenera, genera, and groups of higher rank, the valid name is the earliest name published, provided that this is in conformity with the Rules of Nomenclature.

In species the valid name is the binary and in subspecies the ternary combination containing the earliest epithet published, provided that this combination is in conformity to the Rules of Nomenclature.

**Principle 10.** Bacteriologists are urged not to change a name (or combination of names) without serious motives, based either on more profound knowledge of facts or on the necessity of giving up a nomenclature that is contrary to the Rules.

**Principle 11.** The application of names of taxonomic groups is determined by means of *nomenclatural types*. A nomenclatural type is that constituent element of a group to which the name of the group is permanently attached, whether as an accepted name or as a synonym. The name of a group must be changed if the type bearing that name is excluded.

The type of a generic name is a species, that of the name of a species or subspecies (variety) is usually an authentic culture, a specimen, or a preparation. In some species, however, the type is a description or a figure given by a previous author. Where permanent preservation of a culture, a specimen, or preparation is impossible, the application of the name of a species or subdivision of a species is determined by means of the original description or figure.

(See *Rule 9; Recommendations 9a-d.*)

*Note.* The nomenclatural type is not necessarily the most typical or representative element of a group; it is merely that element with which the name of the group is permanently associated.

(Examples: The type of the name *Bacillus* is the species *Bacillus subtilis*. The type of *Pseudomonas suaveolens* Soppeland is the culture designated and deposited by the author as the type culture in the American Type Culture Collection. The type of *Actinomyces cameli* (Mason) Ford consists of the description and illustration (*J. trop. med. (Ther.)*, 1919, 32, 34) as no cultures are available.)

*Principle 12.* A name of a taxonomic group has no status under the Rules, and no claim to recognition by bacteriologists, unless it is validly published.

(See *Rules 10-14; Recommendations 12a-c.*)

### CHAPTER 3

## RULES OF NOMENCLATURE WITH RECOMMENDATIONS

### Section 1. *Naming of groups of various ranks*

*Rule 1.* Names of divisions, subdivisions, classes, subclasses, orders, suborders, families, subfamilies, tribes and subtribes are taken either from their chief characters or from a taxonomic unit of the next lower rank.

*Rule 2.* The names of all ranks higher than the genus are written in the plural number.

*Rule 3.* Names of divisions, subdivisions, classes, and subclasses are words of Greek or Latin origin.

(See *Principles 4, 5, 7; Rules 24, 25.*)

*Rule 4.* Names of orders, suborders, families, subfamilies, tribes and subtribes are also words of Greek or Latin origin or Latinized words, each with a suffix to indicate its taxonomic rank. The suffix for orders is *-ales*, for suborders *-ineae*, for families *-aceae*, for subfamilies *-oideae*, for tribes *-eae*, and for subtribes *-inae*.

(See *Principles 4, 5, 7; Rules 22, 24, 25.*)

*Rule 5.* Names of genera and of subgenera are substantives (or adjectives used as substantives) in the singular number and written with an initial capital. These names may be taken from any source whatever and may even be composed in an arbitrary manner. They are treated as Latin substantives. Generic names and subgeneric names are subject to the same rules and recommendations and, from a nomenclatural standpoint, they are co-ordinate.

(See *Principles 4, 5, 7.*)

Examples: *Bacillus*, *Pasteuria*, *Brucella*, *Alcaligenes*, *Fusiformis*.

If a genus is divided into subgenera, one of the subgenera (that which includes the type of the genus) should bear the same name as the genus.

(See *Rules 9, 19, 20, 27, 28; Recommendations 9a-c, 19a, 17i.*)

Example: If the genus *Bacillus* is divided into two or more subgenera, the subgenus which includes the type species *Bacillus subtilis* should bear the subgeneric name *Bacillus*.

**Recommendation 5a.** Bacteriologists who are forming new generic or subgeneric names should attend to the following recommendations:

- (1) Not to make names very long or difficult to pronounce.
- (2) To take names that have an agreeable form readily adaptable to the Latin tongue.
- (3) Not to dedicate genera to persons quite unconnected with bacteriology or at least with natural science nor to persons quite unknown.
- (4) To avoid adjectives used as nouns.
- (5) Not to make names by combining words from different languages (*nomina hybrida*).
- (6) To give a feminine form to all personal generic names, whether they commemorate a man or a woman.

(See *Principles* 4, 7; *Rule* 27; *Recommendations* 27a-i for orthography and gender of generic names.)

**Rule 6.** Names of species are binary combinations consisting of the name of the genus followed by a single specific epithet.\* If an epithet consists of two or more words, these must either be united or joined by hyphens. Specific epithets are:

(a) Adjectives, which must agree grammatically with the generic name. Examples: *Bacillus subtilis*, *Micrococcus aureus*, *Clostridium botulinum*.

(b) Substantives, in the nominative, in apposition with the generic name. Examples: *Flavobacterium ceramicola*, *Vibrio comma*, *Pseudomonas conjac*, *Phytomonas holcicola*.

(c) Substantives in the genitive. Examples: *Phytomonas vascularum*, *Aerobacter cloacae*, *Rhizobium leguminosarum*, *Brucella abortus*, *Acetobacter aceti*, *Salmonella anatis*, *Borrelia kochii*.

Within the same genus, no two species names bear the same specific epithet.

(See *Principles* 4, 5, 7; *Rule* 27; and *Recommendations* 27a-i for orthography and gender of specific names.)

**Recommendation 6a.** When it is desired to indicate the name of a subgenus in connection with the generic name and specific epithet, the name of the subgenus may be placed in parentheses between the two.

Example: *Lactobacillus (Thermobacterium) caucasicus*.

(See *Principles* 4, 5, 7.)

**Recommendation 6b.** In forming specific epithets bacteriologists should attend to the following recommendations:

- (1) To choose a specific epithet which, in general, gives some indication of the

\* The term 'epithet' as here used implies a single descriptive word or a single descriptive phrase.

Examples: The Latin word *aureus* (golden) is a single descriptive adjective or epithet, and the species name *Micrococcus aureus* would be in correct form. The phrase *lac acidum* (sour milk) is a single epithet, and the species name *Streptococcus lactis-acidi* (or *lactisacidi*) (*Streptococcus* of sour milk) is in correct form. Care should be used not to regard a sequence of unrelated words as a single epithet. The species name *Bacillus aureus lactis* (the golden bacillus of milk) would be an invalid trinomial; there are two specific epithets. The name cannot be validated by hyphenating the two words as *Bacillus aureus-lactis*; there are still two unrelated epithets. If the two words are combined as in the specific name *Bacillus aurei-lactis*, the meaning is completely changed to *Bacillus* of golden milk; the species name is in correct form, but the meaning is nonsensical unless applied to an organism which changes the colour of the milk to golden.

appearance, the characters, the origin, the history, or the properties of the species. If taken from the name of a person, it usually recalls the name of the one who discovered or described it, or was in some way concerned with it.

Examples: *Micrococcus aureus*, *Clostridium pasteurianum*, *Phytomonas campestris*, *Bacillus viscosus*, *Kurthia zopfii*.

(2) To avoid those which are very long and difficult to pronounce.

(8) To avoid those which express a character common to all or nearly all the species of a genus.

Example: *Micrococcus sphericus*.

(4) To avoid using the names of little-known or very restricted localities, unless the species is quite local.

(5) To avoid, in the same genus, epithets which are very much alike, especially those which differ only in their last letters.

(6) Not to adopt unpublished names found in authors' notes, attributing them to their authors, unless these have approved publication.

(See *Principles* 4, 5, 7.)

**Recommendation 6c.** Names of men and women and also of countries and localities used as specific epithets may be substantives in the genitive (*welchii*) or adjectives (*pasteurianum*, *japonicum*). It will be well, in the future, to avoid the use of the genitive and the adjectival form of the same epithet to designate two different species of the same genus.

(See *Principles* 4, 5, 7.)

**Rule 7.** Names of subspecies (varieties) are ternary combinations consisting of the name of the genus followed by the specific and subspecific epithets in order.

Example: *Escherichia coli* subsp. *communior* (Topley and Wilson) Breed *et al.* 'or *Escherichia coli* var. *communior*, or *Escherichia coli communior*. This does not justify the name *Bacillus fluorescens liquefaciens*, as this name was originally proposed as a trinomial name for a species, and not for a subspecies or a variety.

Epithets of subspecies (varieties) are formed like those of species; when adjectival in form and not used as substantives they agree in gender with the generic name.

Neither within the same species nor within the same genus may two subspecies bear the same subspecific epithet.

If the species is divided into subspecies, the subspecific epithet of the subspecies containing the type of the species shall be the same as that of the species.

(See *Principles* 4, 5, 7.)

Example: If *Micrococcus aureus* is divided into two or more subspecies, one (that containing the type) should be designated *Micrococcus aureus* subsp. *aureus*.

**Rule 8.** Subdivisions of species (other than subspecies (varieties)) are given vernacular names or designated by numerals or letters or, in special cases, are given names in Latin form.

(See *Principles* 4, 5, 7.)

**Recommendation 8a.** Authors of names of subdivisions of species of bacteria which are not treated as subspecies (varieties) should attend to the following recommendations and definitions:

(1) A *strain* is a pure culture of bacteria made up of the descendants of a single isolation. It is frequently designated by the name of the individual responsible for its isolation, as *Corynebacterium diphtheriae* strain Park-Williams. It may also be

designated by the locality or by a number or some similar laboratory distinguishing mark. Strain may also be used to designate cultures of bacteria which correspond to cultivated 'varieties' of higher plants in having some special economic significance. Such are frequently names from the laboratory or factory where isolated, as *Acetobacter aceti* strain Carlsberg.

(2) *Type* is a term which has frequently been used to designate a subdivision of a species, particularly in cases where the differentiating characters are regarded as insufficient to justify the erection of a subspecies or variety. Types are often differentiated on the basis of antigenic characteristics. Type is sometimes used to designate a physiological or morphological variant. In view of the use of the word 'type' in a different sense as defined in Principle 11, it is suggested that the terms *serotype* (or *serological type*), *biotype* (or *physiological type*) and *morphotype* (or *morphological type*) may appropriately be substituted for *type* as a designation of a subdivision of a species.

(3) The term *group* in bacteriology should be used with great care so as to avoid ambiguity. It is employed popularly to designate various organisms with common characteristics (i.e., 'Coli-aerogenes Group') and, in a restricted sense, in antigenic analysis for designating species or subgenera (e.g. *Streptococcus*, group A, Lancefield), or varieties or subspecies (e.g. *Neisseria intracellularis*, group I, Scott). It is suggested that the term *group* be reserved for primary serological divisions and designated by capital letters. Any serological subdivisions within the group should be designated as *types* and distinguished by Arabic numerals (e.g. *Bacterium pseudotuberculosis-rodentium* group A, type 1, Schütze).\*

(4) The designation *phase* should be restricted to use for bacteria showing certain alternative immunologic characteristics, and particularly for the 'specific phase' or 'nonspecific phase' of Andrewes as recorded for the genus *Salmonella*.

Example: *Salmonella enteritidis* specific phase.

(5) A form (*forma*) or special form (*forma specialis*) is a subdivision of a species of a parasitic microorganism distinguished primarily by adaptation to a particular host. It is named preferably by giving it the scientific name of the host. This is written preferably in the genitive.

Example: *Rhizobium phaseoli* forma *phaseoli multiflori* or *Rhizobium phaseoli* f. *phaseoli multiflori*.

(6) A *variant* is an organism showing some variation in some character from the parent culture. Frequently variants result by mutation. If sufficiently distinct and stable, the variant may even be regarded and named as a subspecies or variety. Example of variant: the progeny of a colour sector in a pigmented colony, or the progeny of secondary colonies as lactose-fermenting mutants in colonies of glucose-fermenting bacteria.

Example: *Shigella sonnei* lactose-positive variant.

(7) A *stage* or *state* is the name given to the Rough, Smooth, Mucoid, and similar variants which arise from colonies of many species of bacteria. These are regarded as alternating stages which are generally reversible and indeed by some authors as

\* It may be urged that if specific names are substituted for group letters at present employed (as strictly speaking they should be) the whole serological concept of the genus is obscured, and it would be difficult to get serologists, working in many of the various fields, to assent to an action that they might consider retrograde. But this procedure has been accepted by the Salmonella Subcommittee in the case of the genus *Salmonella*. However, all workers in this field are familiar with the Kauffmann-White schema which clearly shows the antigenic relations within the genus concept. A somewhat similar procedure might be employed, for example, in the case of the streptococci, i.e. an approved schema drawn up showing the antigenic relationships within the various groups which should be given specific rank with names consistent, so far as possible, with the laws of priority. It is suggested that the Salmonella Subcommittee in this regard has made a sincere effort to reconcile the traditions of the past with the present practical necessity of stressing serological relationships, where they exist.

part of a pleomorphic life cycle. They may be designated by some vernacular descriptive name.

Example: *Bacillus subtilis* Rough stage.

(See *Principles* 4, 5, 7b.)

## Section 2. Designation of nomenclatural types

**Rule 9.** For each valid name of each taxonomic group there should be designated a type; that is for each species or subspecies a type culture, specimen, or description, for each genus a type species (genotype).

(See *Principle* 11; *Rule* 5.)

**Recommendation 9a.** When publishing names of new taxonomic groups, authors should indicate carefully the subdivision which is the type of the new name: The type species (genotype) in a genus, the type subspecies or variety in a species in which these subdivisions are recognized, the type specimen, preparation, or description in a species. This type determines the application of the name in the event of the taxonomic group being subsequently divided. When describing new species, varieties, or forms of parasitic bacteria, the host of the type should be indicated.

(See *Principle* 11; *Rule* 5.)

**Recommendation 9b.** When revising a genus for which no genotype has been designated, an author should state which species he accepts as the nomenclatural type.

(See *Principle* 11; *Rule* 5.)

**Recommendation 9c.** In selecting a nomenclatural type (genotype) for a genus of bacteria, bacteriologists should, when possible, choose a species that will fix the generic name as it is now commonly applied.

(See *Principles* 2, 11; *Rule* 5.)

**Recommendation 9d.** The utmost importance should be given to the preservation of the original ('type') material on which the description of the new group is based. The original account should state where this material is to be found. When a new species or subspecies of bacterium is described, if the organism is one which may be maintained in pure culture, an authentic culture labelled as 'type' should be deposited with one of the recognized national or international type culture collections. The national or international type culture depositories recognized are designated by the action of the International Committee of Nomenclature. These recognized in 1939 are the National Collection of Type Cultures of Microorganisms maintained in London, England,\* and the American Type Culture Collection, Washington, D.C. Inasmuch as the type of a bacterial species is frequently the published description and drawings, these should be as complete as possible.

**Note.** It should be borne in mind that morphological, biochemical and antigenic changes, and also loss of virulence, may take place as the result of repeated subculture in the collection. This can to some extent be obviated by drying cultures in high vacuum under optimal conditions and storing them for future reference.

(See *Principle* 11.)

## Section 3. Publication of names

**Rule 10.** Legitimate bacteriological nomenclature begins with Linnaeus' *Species Plantarum*, ed. 1, 1753.†

(See *Principle* 12.)

**Rule 11.** Publication is effected, under these Rules, by sale or distribution of printed matter to the general public or to bacteriological institutions. No

\* Present address: Central Public Health Laboratory, Colindale Avenue, London, N.W. 9.

† Fixed by action of the First International Congress of Microbiology in Plenary Session, Paris, 1930 (*Proceedings*, Part 2, p. 527).

other kind of publication is accepted as *effective* (*effective publication*); communication of new names at a public meeting, or the placing of names in collections, does not constitute effective publication.

Where reprints or separates from periodicals or other works are placed on sale or issued in advance, the date on the separate is accepted as the date of effective publication.

The date of acceptance of an article for publication as given in a publication does not indicate the effective date of publication and has no significance in determination of priority of publication of names.

(See *Principle 12*; *Rule 12*.)

**Rule 12.** A name of a taxonomic group is not validly published unless it is both (1) effectively published (See *Rule 11*), and (2) accompanied by a description of the group or by a reference to a previously and effectively published description of it.

The words 'valid' or 'validly published' as used in these Rules mean 'with standing in nomenclature', and the words 'invalid' or 'not validly published' mean 'without standing in nomenclature'.

Mention of a name on a label on a culture or preparation of bacteria in a collection without printed or autographed description does not constitute valid publication of that name.

A name of a taxonomic group is validly published only if it has been definitely accepted by the author who published it. A name proposed provisionally (*nomen provisorium*) in anticipation of the eventual acceptance of the group, or of the circumscription, position, or rank given to a group, or mentioned only incidentally is not validly published.

Example: Beijerinck (*Arch. neerl. d. sc. exactes*, 1908; Sec. 2, 8, 217) mentioned in a footnote to his article describing and naming the genus *Azotobacter* that *Parachromatium* might be a suitable name. It was never formally proposed or adopted, and has no standing in nomenclature.

A name of a taxonomic group is not validly published when it is merely cited as a synonym.

Example: Trevisan (*Rendiconti Real. Ist. Lombard. d. Sci. e Lett.*, Ser. 2, 1879, 12, 144) cited *Malleomyces equestris* Hallier as a synonym of *Micrococcus equestris*, which he regarded as the causal organism of glanders. Inasmuch as all of Hallier's species were based upon mixed cultures and his names invalid, this incidental citation as synonym by Trevisan does not validate the name. *Malleomyces* must date as a generic name from its proposal by Pribram in 1933 (*Klassifikation des Schizomyceten*, p. 98).

A group is not characterized, and the publication of its name is not validated merely by mention of the subordinate groups included in it: thus, the publication of the name of an order is not validated by mention of the included families; that of a family is not validated by mention of the included genera; that of a genus is not validated by mention of the included species.

The date of a name or of an epithet is that of its valid publication. For purposes of priority, however, only legitimate names and epithets published in legitimate combinations are taken into consideration. In the absence of

proof to the contrary, the date given in the work containing the name or epithet must be regarded as correct.

(See *Principle 12*; *Rule 27*, Note 1.)

**Example:** *Chondromyces crocatus* Berkeley & Curtis 1857 (in Berkeley, *Introduction to Cryptogamic Botany*, p. 313) is a name appended to an illustration with description. The description was published later (Berkeley, *Grevillea*, 1874, 3, 64) and valid publication was of the later date.

**Recommendation 12a.** When publishing names of new groups of bacteria in works written in a language unfamiliar to the majority of workers in bacteriology, it is recommended that the authors publish simultaneously the diagnoses in a more familiar language.

(See *Principle 12*.)

**Recommendation 12b.** Authors should indicate precisely the date of their works. In the case of a work appearing in parts, the last published sheet of the volume should indicate the precise dates on which the different fascicles or parts of the volume were published as well as the number of pages in each.

(See *Principle 12*.)

**Recommendation 12c.** When works are published in periodicals, the author should require the publisher to indicate on the separates or reprints the date (year and month, if possible the day) of publication and also the title of the periodical from which the work is extracted. Separates or reprints should always bear pagination of the periodical of which they form a part; if desired, they may also bear a special pagination.

(See *Principle 12*.)

**Rule 13.** A name of a genus is not validly published unless it is accompanied (1) by a description of the genus, or (2) by the citation of a previously and effectively published description of the genus under another name; or (3) by a reference to a previously and effectively published description of the genus as a subgenus, or other subdivision of a genus.

The name of a monotypic new genus based on a new species is validated by the provision of a combined generic and specific description.

(See *Principle 12*.)

**Examples of validly published generic names:** *Bacillus* Cohn 1872, *Pasteurella* Trevisan 1885, *Sarcina* Goodsir 1842, *Polyangium* Link 1809.

**Rule 14.** The name of a species or a subspecies (variety) is not validly published unless it is accompanied (1) by a description of the group; or (2) by the citation of a previously and effectively published description of the group under another name.

(See *Principle 12*.)

**Example of validly published name of species:** *Bacillus subtilis* Cohn 1872.

#### Section 4. Citation of authors and names

**Rule 15.** For the indication of the name (unitary, binary, or ternary) of a group to be accurate and complete, and in order that the date may be readily verified, it is necessary to cite the author who first published the name in question.

**Examples:** *Plocamobacteriales* Pribram (or Pribram 1933), *Proteus* Hauser (or Hauser 1885), *Serratia marcescens* Bizio (or Bizio 1823).



An alteration of the diagnostic characters or of the circumscription of a group without exclusion of the type does not warrant the citation of an author other than the one who first published the name. When the changes have been considerable, an indication of their nature and of the author responsible for the change is added, as, *em.* (*emendavit*) or *mutatis charact.*, or *pro parte*, or *excl. gen.*, *excl. sp.*, *excl. var.*, or some other abridged indication.

Example: *Bacillus* Cohn *em.* Migula.

When a name of a taxonomic group has been proposed but not published by one author, and is subsequently validly published and ascribed to him (or her) by another author who supplied the description, the name of the latter author must be appended to the citation with the connecting word *ex*. If it is desirable or necessary to abbreviate such a citation, the name of the publishing author, being the more important, must be retained.

Example: *Salmonella dar-es-salaam* Schütze *ex*. Brown, Duncan and Henry.

When a name and description by one author are published by another author, the word *apud* is used to connect the names of the two authors, except where the name of the second author forms part of the title of a book or periodical, in which case the connecting word *in* is used instead.

**Rule 16.** When a genus, a subgenus, a species, or a subspecies (variety) is altered in rank but retains its name or epithet, the original author must be cited in parentheses, followed by the name of the author who effected the alteration. The same holds when a subgenus, a species, or a subspecies (variety) is transferred to another genus or species with or without alteration of rank.

Example: *Spirochaete pallida* Schaudinn and Hoffman becomes *Treponema pallidum* (Schaudinn and Hoffman) Schaudinn.

**Recommendation 16a.** When citing a name published as a synonym, the words 'as synonym' or 'pro synon.' should be added to the citation.

When an author publishes as a synonym a manuscript name of another author, the word *ex* should be used to connect the names of the two authors.

**Recommendation 16b.** When citing in synonymy a name invalidated by an earlier homonym, the citation should be followed by the name of the author of the earlier homonym preceded by the word 'non', preferably with the date of publication added. In some instances it will be advisable to cite also any later homonym or homonyms.

Example: *Myxococcus* Gonnerman 1907 *non* Thaxter 1892.

## Section 5. *Changes in names as a result of segregation or union of groups or change in rank of groups*

**Rule 17.** An alteration of the diagnostic characters, or of the circumscription of a group, does not warrant a change in its name except insofar as this may be necessitated (1) by transference of the group or (2) by a change of its rank.

When a genus is divided into two or more genera, the generic name must be retained for one of them, or (if it has not been retained) must be re-established. When a particular species was originally designated as the type, the generic name must be retained for the genus including that species. When no type was designated, a type must be chosen.

**Example:** Donker (1926) divided the genus *Bacillus* into *Bacillus* and *Aerobacillus*, retaining *Bacillus* for the genus containing the type species *Bacillus subtilis*.

The same rule is applied when a subgenus is divided into two or more subgenera.

**Rule 18.** When a species is divided into two or more species, the specific epithet must be retained for one of them, or (if it has not been retained) must be re-established. When a particular specimen was originally designated as the type, the specific epithet must be retained for the species including that specimen. When no type was designated, a type must be chosen according to the regulations given.

The same rule applies to subspecies (varieties); for example, to a subspecies (variety) divided into two or more subspecies (varieties).

**Example:** When *Rhizobium leguminosarum* Frank was divided into several species, all symbiotic on the roots of leguminous plants, the name *R. leguminosarum* was correctly retained for one of them by Fred.

When a species is transferred to another genus (or placed under another generic name for the same genus), without change of rank, the specific epithet must be retained or (if it has not been retained) must be re-established unless one of the following obstacles exists: (1) the resulting binary name is a later homonym or tautonym or (2) there is available an earlier validly published specific epithet.

When the specific epithet, on transference to another generic name, has been applied erroneously in its new position to a different species, the new combination must be retained for the organism on which the epithet was originally based.

**Rule 19.** When two or more groups of the same rank are united, the oldest legitimate name or (in species and their subdivisions) the oldest legitimate epithet is retained. If the names or epithets are of the same date, the author who unites the group has the right of choosing one of them. The author who first adopts one of them, definitely treating another as a synonym or referring it to a subordinate group, must be followed.

(See *Rule 5*.)

**Recommendation 19a.** Authors who have to choose between two generic names should note the following recommendations:

(1) Of two names of the same date to prefer the one which was first accompanied by the description of a species.

(2) Of two names of the same date, both accompanied by descriptions of species, to prefer the one which, when the author made his choice, included the larger number of species.

(3) In cases of equality from these various points of view to prefer the more correct and appropriate name.

(See *Rule 5*.)

**Rule 20.** When several genera are united as subgenera under one generic name, the subgenus including the type of the generic name used must bear that name unaltered.

(See *Rule 5*.)

**Rule 21.** When several species are united as subspecies or varieties under one specific name, the subdivision which included the type of the specific epithet used must be designated by the same epithet unaltered.

**Rule 22.** (1) When a subtribe becomes a tribe, when a tribe becomes a subfamily, when a subfamily becomes a family, etc., or when the inverse changes occur, the stem of the name should not be altered but only the termination (-inae, -cae, -oideae, -aceae, -ineae, -ales, etc.).

(2) When a subgenus becomes a genus, or the inverse changes occur, the original name should be retained.

(8) When a subdivision of a species becomes a species, or the inverse change occurs, the original epithet should be retained unless the resulting combination is rejected under Section 6.

(See *Rules 3, 4.*)

#### Section 6. *Rejection and replacement of names*

**Rule 28.** A name or epithet must not be rejected, changed, or modified merely because it is badly chosen or disagreeable, or because another is preferable or better known.

(See *Principles 1, 8, 10.*)

**Rule 24.** A name must be rejected if it is illegitimate, i.e. if it is contrary to a rule. The publication of an epithet in an illegitimate combination must not be taken into consideration for purposes of priority.

(See *Principle 1 Rules 1-4.*)

A name of a taxonomic group is illegitimate in the following cases:

(1) If it was nomenclaturally superfluous when published, i.e. if the group to which it was applied, as circumscribed by its author, included the type of a name which the author ought to have adopted under one or more of the Rules.

Example: *Dicrobacterium* Enderlein 1917 was superfluous because of the previous publication of *Serratia* Bizio 1828. •

(2) If it is a binary or ternary name published in contravention of *Principle 9* and *Rules 17-28*, i.e. if its author did not adopt the earliest legitimate epithet available for the group with its particular circumscription, position, and rank.

(8) If its specific epithet must be rejected under *Rule 25*.

(4) If it is a later homonym of a genus of bacteria, of a genus of plants, or of a genus of protozoa; that is, if it duplicates a name previously and validly published for a group of the same rank based on a different type. Even if the earlier homonym is illegitimate, or is generally treated as a synonym on taxonomic grounds, the later homonym must be rejected. When an author simultaneously publishes the same new name for more than one group, the first author who adopts one of them, or substitutes another name for one of them, must be followed.

**Recommendation 24a.** Authors should avoid introducing into bacteriology as generic names such names as are in use in zoology.

(See *Principle 5.*)

**Note.** Mere orthographic variants of the same name are treated as homonyms when they are based on different types.

(See *Rule 28.*)

(5) If, owing to a segregation, it is used with different meanings, and so becomes a permanent source of confusion or error. A list of names to be abandoned for this reason will be included under *nomina rejicienda*.

(See *Principle 3; Provision 3.*)

(6) If its application is uncertain (*nomen dubium*). A list of names to be abandoned for this reason will be included under *nomina rejicienda*.

(See *Provision 3.*)

(7) If the characterization of the group was based upon an impure or mixed culture. A list of names to be abandoned for this reason (*nomina confusa*) will be included under *nomina rejicienda*.

(See *Provision 3.*)

Examples: The characters of the genus *Malleomyces* Hallier 1870 were derived from various fungi and bacteria erroneously supposed to be growth forms of a single organism. The name *Salmonella tokio* Aoki was based upon a mixed culture.

(8) If it was based upon an abnormality.

Example: An eroded colony of *Shigella dysenteriae* due to bacteriophage action would be such an abnormality.

**Rule 25.** Specific epithets are illegitimate in the following special cases and must be rejected.

(1) When they are merely words not intended as names.

(2) When they are merely ordinal adjectives being used for enumeration.

(3) When they exactly repeat the generic name (Tautonym).

(See *Principles 1, 9; Rules 1, 2.*)

**Rule 26.** The name or epithet to be rejected according to *Rules 23–25* is replaced by the oldest legitimate name, or (in a combination) by the oldest legitimate epithet which will be, in the new position, in accordance with the Rules. If none exists, a new name or epithet must be chosen. Where a new epithet is required, an author may, if he wishes, adopt an epithet previously given to the group in an illegitimate combination, if there is no obstacle to its employment in the new position or sense.

(See *Principles 1, 9.*)

## Section 7. Orthography and gender of names

**Rule 27.** The original spelling of a name or epithet must be retained, except in the case of a typographical error, or of a clearly unintentional orthographic error. When the difference between two generic names lies in the termination, these names must be regarded as distinct, even though differing by one letter only. This does not apply to mere orthographic variants of the same name.

(See *Principles 1, 4, 9; Rules 5, 6 (c); Recommendation 5a.*)

Example: *Streptococcus erysipelatos* and *S. erysipelatis* are mere orthographic variants of the same name. *Erysipelatos* is the strict transliteration of the Greek genitive; *erysipelatis* is the form of the more usual and preferable transliteration into Latin form.

**Note 1.** The words 'original spelling' in this Article mean the spelling employed when the name was validly published.

(See *Rules 1, 2.*)

**Note 2.** The use of a wrong connecting vowel or vowels (or the omission of a connecting vowel) in a specific epithet, or in the name of a genus is treated as an unintentional orthographic error which may be corrected.

**Note 3.** In deciding whether two or more slightly different names should be treated as distinct or as orthographic variants, the essential consideration is whether they may be confused with one another or not. If there is a serious risk of confusion they should be treated as orthographic variants. Doubtful cases should be referred to the Judicial Commission for an Opinion.

**Note 4.** Specific and other epithets and names of Greek origin differing merely by having Greek and Latin terminations respectively are orthographic variants. Epithets bearing the same meaning and differing only slightly in form are considered as orthographic variants. The genitive and adjectival forms of a personal name are, however, treated as different epithets.

Example: *Hormodendron* and *Hormodendrum*; the strict transliteration of the Greek neuter ending is *-on*. The usual and preferable transliteration into the Latin is *-um*.

The liberty of correcting a name must be used with reserve, especially if the change affects the first syllable, and above all the first letter of the name.

**Recommendation 27a.** When a new name is derived from a Greek work containing the *spiritus asper* (rough breathing), this should be transcribed as the letter *h*.

(See *Principles* 1, 4; *Recommendation* 5a; *Rule* 6 (c).)

**Recommendation 27b.** For scientific names it is advisable to use another font than that used for the remainder of the text, or to space the letters, or to use similar device appropriate to the text.

Example: 'The disease anthrax is caused by *Bacillus anthracis* Koch.' Typewritten scientific names should be underlined.

(See *Principles* 1, 4; *Recommendation* 5a; *Rule* 6 (c).)

**Recommendation 27c.** When a new name for a genus or subgenus is taken from the name of a person, it should be formed in the following manner:

(1) When the name of the person ends in a vowel the letter *a* is added (thus, *Gaffkya* after Gaffky; *Noguchia* after Noguchi; *Serratia* after Serrati), except when the name already ends in *a*, when *ea* is added (e.g. *Collaea* after Colla).

(2) When the name of a person ends in a consonant the letters *ia* are added (e.g. *Escherichia* after Escherich, *Erwinia* after Erwin F. Smith, *Pasteuria* after Pasteur), except when the name ends in *er*, when *a* is added (e.g. *Kernera* after Kerner).

(3) Names may be formed by use of a prefix or a suffix, or modified by anagram or abbreviation. In these cases they count as different words from the original name.

In many cases the names of bacterial genera are formed from the names of persons by the addition of a diminutive ending. The most common modern Latin convention is to add one of the endings *-ellus*, *a*, *um*, preferably *-ella* to conform to *Recommendation* 5a. In some few cases one of the endings *-illus*, *a*, *um* has been added.

(See *Principles*, 1, 4; *Recommendation* 5a; *Rule* 6 (c).)

(4) The syllables which are not modified by these endings retain their original spelling, even with the consonants *k* and *w* or with the groupings of vowels which were not used in classical Latin. Letters foreign to botanical Latin should be transcribed and diacritic signs suppressed. The Germanic *ä*, *ö*, *ü* become *ae*, *oe*, *ue*; the French *é*, *è*, and *ê* become generally *e*. In works in which diphthongs are not represented by special type, the diaeresis sign should be used where required, e.g. *Aerobacillus* not *Aerobacillus*.

**Recommendation 27d.** A new specific or subspecific (varietal) epithet taken from the name of a man may assume either a substantival or an adjectival form. The syllables which are not modified by these endings retain their original spelling, even with the consonants *k* or *w* or with the groupings of vowels which were not used in classical Latin. Letters foreign to botanical Latin should be transcribed and

diacritic signs suppressed. The Germanic *ä, ö, ü* become *ae, oe, ue*. The French *é, è, ê* become generally *e*.

When the epithet is a substantive, it is formed in the following manner:

(1) When the name of the person ends in a vowel, the letter *i* is added (thus, *sonnei* from *Sonne*) except when the name ends in *a*, when *e* is added (thus, *balansae* from *Balansa*).

(2) When the name ends in a consonant, the letters *ii* are added (thus, *welchii* from *Welch*) except when the name ends in *-er*, when *i* is given (thus, *barkeri* from *Barker*).

When the epithet is an adjective, it is formed by the addition of an appropriate ending (thus, *pasteurianus, a, um* from *Pasteur*).

(See *Principles 1, 4; Recommendations 5a; Rule 6 (c).*)

**Recommendation 27e.** The same provisions apply to epithets formed from the names of women. When these have a substantival form they are given a feminine termination. (Thus, *Cytophaga krzemieniewskae*.)

(See *Principles 1, 4; Recommendation 5a; Rule 6 (c).*)

**Recommendation 27f.** New specific (or other) epithets should be written in conformity with the original spelling of the words from which they are derived and in accordance with the rules of Latin and latinization.

Examples: *silvestris* (not *sylvestris*), *sinensis* (not *chinensis*).

(See *Principles 1, 4; Recommendation 5a; Rule 6 (c).*)

**Recommendation 27g.** Specific epithets, even those derived from names of persons, should not be capitalized.

(See *Principles 1, 4; Recommendation 5a; Rule 6 (c).*)

**Recommendation 27h.** In the formation of names or epithets composed of two or several roots taken from Latin or Greek, the vowel placed between the two roots becomes a connecting vowel, in Latin usually *i*, in Greek usually *o*. When the second root begins with a vowel and euphony requires, the connecting vowel should be eliminated (e.g. *lepidantha*). The connecting vowels *ae* should be retained only where this is required for etymological reasons (e.g. *caricaeformis* from *Carica*, in order to avoid confusion with *cariciformis* from *Carex*). In certain compounds of Greek words, no connecting vowel is required, e.g. *brachycarpus* and *glycyphyllus*.

(See *Principles 1, 4; Recommendation 5a; Rule 6 (c).*)

**Recommendation 27i.** Authors should give the etymology of new generic names, and also of new epithets when the meaning of these is not obvious.

(See *Principles 1, 4; Rule 5; Recommendation 5a; Rule 6 (c).*)

**Rule 28.** The gender of generic names is governed by the following regulations:

(1) A Greek or Latin word adopted as a generic name retains its classical gender. In cases where the classical gender varies the author has the right of choice between the alternative genders. In doubtful cases general usage should be followed.

(2) Generic names which are modern compounds formed from two or more Greek or Latin words take the gender of the last. If the ending is altered, however, the gender will follow it.

Example: *Spirochaete* is feminine because the Greek noun *chaete* (χαίτη) is feminine. However, if a name *Spirochaetum* were proposed it would be neuter.

(3) Arbitrarily formed generic names or vernacular names used as generic names take the gender assigned to them by their authors. Where the original author has failed to indicate the gender, the next subsequent author has the right of choice.

(See *Principle 4; Rule 5.*)

## CHAPTER 4

## PROVISIONS FOR EXCEPTIONS TO THE RULES AND FOR THE INTERPRETATION AND MODIFICATION OF RULES

*Provision 1. Modification and amendment of Rules.* These Rules can be amended only by action of a plenary session of an International Congress for Microbiology convened by the International Association of Microbiologists.

*Provision 2. Lists of *nomina conservanda*.* To avoid disadvantageous changes in the nomenclature of the genera by the strict application of the Rules of Nomenclature, the Rules provide for a list of names which must be retained as exceptions (*nomina conservanda*).

*Note 1.* This list of conserved names will remain permanently open for additions. Any proposal of an additional name must be accompanied by a detailed statement of the case for and against its conservation. Such proposals must be submitted to the Judicial Commission (see *Provision 4*) for study and appropriate action.

*Note 2.* When a name proposed for conservation has been provisionally approved by the Judicial Commission, bacteriologists are authorized to retain it pending the decision of the next International Congress for Microbiology.

*Note 3.* A conserved name is conserved against all other names for the group, whether these are cited in the corresponding list of rejected names or not, so long as the group concerned is not united with another group bearing a legitimate name. In the event of union or reunion with another group, the earlier of the two competing names is adopted in accordance with *Rules 19, 20, and 21*.

*Note 4.* A conserved name is conserved against all earlier homonyms.

*Example:* The generic name *Bacillus* Cohn with the type species *B. subtilis* Cohn *em. Prazmowski* is conserved by recommendation of the Nomenclature Committee and the action of the Second International Congress for Microbiology.

(See *Principles 6, 9; Rule 24 (5), (6), (7).*)

*Provision 3. Lists of *nomina rejicienda*.* To avoid unnecessary confusion in the nomenclature of bacteria by the strict application of the rules of nomenclature, the Rules provide a list of names (*nomina rejicienda*) which are not to be used, i.e. are to be permanently rejected. This list includes names which, owing to segregation, are used with different meanings and have become a permanent source of confusion or error (*nomina ambigua*), names where application is uncertain (*nomina dubia*), and names applied to a group made up of two or more discordant elements, especially if these elements were erroneously supposed to form part of the same individual (*nomina confusa*).

(See *Principles 6, 9; Rule 24 (5), (6), (7).*)

*Note 1.* This list of rejected names will remain permanently open for additions. Any proposal of an additional name must be accompanied by a detailed statement of the case for and against its rejection. Such proposals must be submitted to the Judicial Commission of the Nomenclature Committee for study and appropriate action. When a name proposed for rejection has been provisionally rejected by the Judicial Commission, bacteriologists are authorized to reject it pending the decision of the next International Congress for Microbiology.

*Note 2.* A rejected name may not be later introduced into bacteriological literature, except that *nomina dubia* may be removed from the list upon submission of evidence of correct status and by action by the Judicial Commission on Nomenclature.

*Provision 4. Authorization of a Nomenclature Committee.* A permanent Nomenclature Committee has been established by the International Association

of Microbiologists in Congress. This Nomenclature Committee is so constituted that wherever practicable each nation is represented by at least one member, and no nation by more than five. Recommendations for nomination for membership on this Nomenclature Committee may be made by any society of microbiologists or by members of any International Congress. Recommendations for nominations should be made to one of the Permanent Secretaries who will present them to the Nomenclature Committee for consideration at its next meeting. Appointments to membership on the Nomenclature Committee are made by nomination by the Nomenclature Committee and election by the next following Plenary Session of an International Congress for Microbiology. The International Congress elects two Permanent Secretaries, one primarily to represent medical bacteriology and one to represent nonmedical bacteriology. The Nomenclature Committee shall elect such other officers as may be desired. A complete list of all members of the Nomenclature Committee shall be published in the *Proceedings* of each triennial meeting of the International Congress for Microbiology.

The Nomenclature Committee selects from its membership a Judicial Commission consisting of twelve members, exclusive of members *ex officio*, and designates a Chairman from the membership of the Commission. The two Permanent Secretaries of the Nomenclature Committee are members *ex officio* of the Judicial Commission. The commissioners serve in three classes of four commissioners each for nine years, so that one class of four commissioners retires at each International Congress. In the event of failure of the International Congress to meet triennially, the term of office of each class will automatically be extended by the number of years greater than three elapsing between successive Congresses. In case of resignation or death of any commissioner, his place shall be filled for the unexpired term by the Nomenclature Committee at its next meeting.

A. The Nomenclature Committee has the following functions:

(1) To consider and pass upon all recommendations relating to the formulation or modification of Rules of Nomenclature, particularly such rules as relate to bacteria, but also pertaining to nomenclature of other groups when desirable. The Committee will recommend such action as may be appropriate to the next Plenary Session of an International Congress for Microbiology.

(2) To consider all Opinions rendered by the Judicial Commission. Such Opinions become final if not rejected at the meeting of the International Committee next following the date on which the Opinion was issued.

(3) To designate official Type Culture Collections.

(4) To receive and act upon all reports and recommendations received from the Judicial Commission or other committees relating to problems of nomenclature or taxonomy.

(5) To hold at least one meeting triennially in connection with the meeting of the International Congress for Microbiology.

(6) To report to the final Plenary Session of each Congress a record of its actions, and to recommend for approval such actions and nominations as require the approval of the Congress.



(7) To co-operate with other Committees, particularly those of the International Botanical and Zoological Congresses, to consider common problems of nomenclature.

(See *General Considerations* 1.)

B. The Judicial Commission of the Nomenclature Committee has the following functions:

(1) To issue formal '*Opinions*' when asked to interpret rules of nomenclature in cases in which the application of a rule is doubtful.

(2) To prepare formal '*Opinions*' relative to the status of names which have been proposed, placing such names when deemed necessary in special lists, such as lists of *nomina conservanda*, *nomina rejicienda*, etc.

(8) To develop recommendations for emendations of the International Rules for Bacteriological Nomenclature, for presentation to the Nomenclature Committee.

(4) To prepare formal '*Opinions*' relative to types, particularly types of species and genera, and to develop a list of bacterial genera which have been proposed with the type species of each.

(5) To prepare and publish lists of names of genera which have been proposed for bacteria, for protozoa, or for other groups in which microbiologists are interested in order to assist authors of new names in avoiding invalid homonyms.

(6) To develop a list of publications in microbiology whose names of organisms shall have no standing in bacteriology in determination of priority.

(7) To edit and publish the International Rules of Bacteriological Nomenclature, *Opinions*, Lists of *Nomina Conservanda*, *Nomina Rejicienda*, Type Species, etc.

(8) To report to the Nomenclature Committee at its triennial meetings all Recommendations, Transactions, and *Opinions*.

(9) To report to the International Committee at its triennial meetings the names of all Commissioners whose terms of service expire, likewise a list of all vacancies caused by resignation or death.

(10) To prepare '*Opinions*' when requested relative to the nomenclatural status of microorganisms studied by microbiological techniques, but not classed with the bacteria or viruses; for example, the yeasts, molds, and protozoa. However, such '*Opinions*' shall not be issued until confirmed by the commission charged with the interpretation of the appropriate code of nomenclature (Botanical or Zoological).

**Recommendation 4.** Whenever, in the opinion of any microbiologist an interpretation of any rule or recommendation of nomenclature is desirable because the correct application of such a rule or recommendation is doubtful, or the stability of nomenclature could be increased by the conservation or by the rejection of some name which is a source of confusion or error, it is recommended that he prepare a résumé outlining the problem, citing pertinent references, and indicating reasons for and against specific interpretations. This résumé should be submitted to the Chairman of the Judicial Commission; if desired, through one of the Permanent Secretaries. An Opinion will be formulated, which may not be issued until it has been approved by at least eight members of the Commission.

(See *Principles* 2, 6.)

## CORRIGENDUM

# The Biological Assay of Streptomycin by a Modified Cylinder Plate Method

By K. A. BROWNLEE, C. S. DELVES, M. DORMAN, C. A. GREEN,  
E. GRENFELL, J. D. A. JOHNSON AND N. SMITH

*J. gen. Microbiol.* (1948), 2, 40.

Mr D. R. Read has pointed out that, in the design given in Table 7, not only is 1 degree of freedom for parallelism confounded with columns but another is confounded with rows. There are accordingly only 5 out of the 7 degrees of freedom for parallelism left unconfounded, and the lower part of the analyses of variance in Table 9 should read as follows:

| Source of variance | Degrees of freedom | Assay no. 8 |         | Assay no. 15 |         | Assay no. 17 |         |
|--------------------|--------------------|-------------|---------|--------------|---------|--------------|---------|
|                    |                    | S.S.        | M.S.    | S.S.         | M.S.    | S.S.         | M.S.    |
| Parallelism        | 5                  | 0.11958     | 0.02391 | 0.02828      | 0.00566 | 0.10063      | 0.02013 |
| Residual           | 36                 | 0.27436     | 0.00762 | 1.17939      | 0.03276 | 0.79124      | 0.02198 |



[The Editors of the Journal of General Microbiology accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.]

## THE SOCIETY FOR GENERAL MICROBIOLOGY

*The Society for General Microbiology held its Seventh General Meeting in the Botany Department of the University of St Andrews on Monday and Tuesday, 18 and 14 September 1948. The following communications were made:*

### COMMUNICATIONS

#### **Growth Requirements of Virulent and Avirulent Strains of *Haemophilus pertussis* in Semisynthetic Medium. By A. JAMES (Greenford)**

The liquid semisynthetic medium of Cohen & Wheeler (1945) was used for the experiments to be described in this communication. Of the eighteen strains of *H. pertussis* tested, starch was found to be essential in the medium for the growth of the fourteen virulent strains, the virulence being estimated by their ability to kill mice after intranasal infection. The four avirulent strains and two strains of *H. parapertussis* grew as readily in the absence as in the presence of starch. The agglutination titre of the virulent organisms with anti-phase I serum after 12 days' growth in the liquid medium was the same as that of the original organisms washed off Bordet-Gengou medium which were used for the inoculum.

A study has been made of the amino-acid requirements of the various strains of *H. pertussis*. The following amino-acids in the medium are completely removed by both virulent and avirulent strains in 12-15 days' growth, as shown by the technique of paper chromatography: aspartic and glutamic acids, serine, glycine and proline; while threonine and alanine are only partially used. The rate of utilization of these amino-acids, as shown by the chromatogram at 5, 7, 9, 12 and 15 days, is greater during the growth of the virulent organisms than during the growth of the avirulent organisms. It was further observed that avirulent organisms used more of the individual amino-acids when grown in medium lacking starch, than in medium containing starch.

It appeared at first that yeast dialysate was essential for the growth of *H. pertussis* in semisynthetic medium, a small amount only being required to give luxuriant growth. Hornibrook (1940) showed that nicotinamide or nicotinic acid could replace the yeast dialysate. This was confirmed and in addition it was shown that cozymase could also replace the yeast dialysate. It thus appears that given the nicotinic acid residue *H. pertussis* is capable of synthesizing the cozymase molecule.

### REFERENCES

- COHEN, S. & WHEELER, M. (1945). *Rep. Div. Lab. & Res. N.Y. State Dep. Hlth*, p. 39.  
HORNIBROOK, J. W. (1940). *Proc. Soc. exp. Biol., N.Y.*, 45, ii, 598.

**The Precipitability of *Haemophilus pertussis* Strains with Aluminium Phosphate and its Significance.** By J. UNGAR and P. MUGGLETON (*Greenford*)

Forty-six strains of *H. pertussis* were tested for their biological properties. Thirty-four strains were recently isolated from cases of pertussis and all of these strains agglutinated by immune antiphase I serum. Out of the twelve laboratory strains, only seven strains were agglutinated with antiphase I serum, five were non-agglutinable. It was found that all the strains grown on Bordet-Gengou medium, which agglutinated with antiphase I serum, were adsorbed on aluminium phosphate, whereas non-agglutinable strains were not precipitated. Strains which were originally agglutinable and precipitable with aluminium phosphate, lost the agglutinability and the precipitability on repeated subcultures. Agglutinable strains are lysed by the addition of N/1-sodium hydroxide or 10 % solution of sodium desoxycholate. Agglutinable strains which are precipitable by the aluminium salt are virulent to mice. In addition, the virulent strains produce toxic substances in the semisynthetic fluid medium, which cause a dermonecrotic reaction in the rabbit's skin. These toxic products are thermolabile, non-filtrable through bacterial filters and can be regarded as disintegrated bacterial particles. These particles are precipitated by aluminium phosphate. We have observed that strains grown on fluid media, which have lost their agglutinability, were no longer virulent to mice, were not precipitated by aluminium phosphate and did not produce toxic substances.

*Summary of difference between strains*

| Property                               | Agglutinable strain<br>(titre over 1/8000 with<br>phase I serum) | Non-agglutinable strain<br>(titre less than 1/500<br>with phase I serum)      |
|--|--|---|
| Precipitation with aluminium phosphate | Complete precipitation   | Not precipitated  |
| Morphology                             | Short Gram-negative bacillus                                     | Shorter and smaller than agglutinable organisms and tends to be Gram-positive |
| Capsulation                            | Capsulated (wide zone noticed after 24 hr.)                      | Capsulated  |
| Solubility in NaOH and bile salt       | Completely soluble   | Insoluble   |
| Virulence in mice                      | Mostly virulent  | Avirulent   |
| Endotoxin production in liquid medium  | Mostly toxin producing   | Non-toxic   |

It is likely that a common factor in the bacterial cell is responsible for the phenomenon of agglutinability, precipitability and virulence in mice. The adsorption by aluminium phosphate is a quick and a convenient method for differentiation between virulent and avirulent *H. pertussis* strains.

**Morphological Evidence in the Taxonomy of the Coccaceae.** By K. A. BISSET (*Birmingham*)

The cocci are morphologically heterogeneous. The *Staphylococci* and *Neisseria* are divisible into two main groups on this ground; the majority of *Staphylococci* and some *Neisseria* possess a structure which is typified in the *Gonococcus*.

The latter does not consist of two cocci, but of a single coccus divided into two cells by a transverse septum. The majority of pathogenic species, of both genera, are of this type. The second group consists of single-celled cocci containing a central nucleus, and never forming transverse septa. Some *Staphylococci*, including a few pathogenic strains, are of this morphology, as are most non-pathogenic *Neisseria*. This type of coccus bears a closer resemblance to certain yeasts and to *Azotobacter*, than to the more typical *Eubacteria*. The two-celled type of coccus resembles the long-chained *Streptococcus*, except that the latter is bilaterally and the former radially symmetrical. The lanceolate type of *Streptococcus* and *Pneumococcus* is in most respects indistinguishable from the rod-shaped *Eubacteria* of smooth morphology, is the only type of coccus in which paired chromosomes can be discerned, and has been observed to undergo a cycle of nuclear changes similar to that found in those rod-shaped genera possessing paired chromosomes.

The fact that long-chained and lanceolate *Streptococci* may be intervariable presumably indicates that the *Streptococci*, together with the two-celled *Staphylococci* and *Neisseria*, are related to the rod-shaped *Eubacteria*. The single-celled cocci are possibly related to the yeasts.

**The Morphology of Sulphate-Reducing Bacteria.** By K. R. BUTLIN, MARY E. ADAMS and MARGARET THOMAS (*Teddington*)

Mesophilic strains show pronounced pleomorphism. Coccoid forms and very short rods were obtained in certain conditions, in addition to the well-known vibrios and spirilla of variable length.

Vibrios almost exclusively predominated in sulphate-lactate-yeast extract medium. Except for one non-flagellated strain, vibrios in young cultures were actively motile, each cell exhibiting variety of movements not easily explained by possession of the single polar flagellum shown by electron micrographs.

Large numbers of spiral cells appeared in cultures grown in sulphate-lactate medium containing 8 %  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ . The effect varied with different strains. The non-motile strain produced spirals and threads up to  $100\mu$  in length. The influence of sulphite was considerably diminished by addition of yeast extract and was reversed by subculture into medium containing no sulphite. None of our observations supports the suggestion in Bergey, Breed, Murray & Hitchen (1948) that the spiral forms consist of short chains.

Our strains produced single-flagellated short rods and coccoid cells in abundance after 8 days' growth in sulphate-lactate-yeast extract medium containing no added ferrous salt in completely filled sealed vessels. Subculture into fresh medium yielded pure cultures of vibrios.

Thermophilic ( $55^\circ$ ) cells are longer, straighter and usually plumper than mesophilic vibrios. They do not produce spirillum forms, though unusually long cells sometimes appear. Electron micrographs show that several flagella are scattered round the cells, which spin, twist and rotate with very little progression.

Some thermophilic and mesophilic strains are inter-convertible by gradual changes in temperature. Starkey (1988) found that the critical temperature at which the morphological change which accompanies the conversions occurs, lies between 40 and 45°. We have been able to confirm and illustrate this change of morphology by means of the electron microscope.

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- STARKEY, R. L. (1988). A study of spore formation and other morphological characteristics of *Vibrio desulphuricans*. *Arch. Mikrobiol.* 9, 268.

#### A Schematic Classification for the Lactic Acid Bacteria.

By J. G. DAVIS (*London*)

The lactic acid bacteria are probably the most homogeneous and clearly defined of any naturally occurring group of organisms.

The group is normally divided into streptococci and lactobacilli on the basis of morphology. These can each be further divided into three clear-cut groups on the basis of cultural characteristics, fermentation and respiration. Not only are these groups closely parallel one to the other but even species within the group are often remarkably similar. The prolonged study of strains isolated from a variety of habitats shows that while most strains can be easily classified and given species names, some fall between the recognized species and sometimes even between subgroups and main groups. Thus, no matter what characteristics are used for differentiation and classification there are always found in practice strains which have intermediate properties. This raises a difficult problem if genus status is given to the three main groups. It is customary to refer to these strains as intermediates and sometimes to ignore them for classification purposes, which is unsatisfactory.

The proposed schematic classification makes provision for these intermediate strains by providing a very elastic method for classification and nomenclature, which is based primarily upon metabolic characteristics (respiration and fermentation). The diagrammatic classification may be considered in either two or three dimensions and consists of three interlacing circles or spheres arranged in such a way that not only does each circle or sphere interlace with each other but all interlace to a slight extent. Thus, a strain or type falling into an interlacing section may be regarded as intermediate to those two and called, for example, I-II, II-III, etc., and strains found to fall in the interlacing of all three may be called I-II-III. To achieve this elasticity generic names have been abandoned inside the group with the exception of *Streptococcus* and *Lactobacillus*.

It is of interest to note that the only marked differences between these two genera are: (i) morphology, (ii) rate of growth, (iii) final acidity in carbohydrate media, and even these sometimes overlap. Thus the separation of the cocci and rods and the placing of other rod genera such as *Microbacterium*, *Propionibacterium*, etc., nearer to *Lactobacillus* than *Streptococcus* in classifications is a violation of the facts.

**A CO<sub>2</sub>-Sensitive Mastitis *Streptococcus*.** By A. CUNNINGHAM, MARGARET A. WILSON and D. H. McLEOD (*Edinburgh*)

Symptoms of a mild but rapidly spreading mastitis appeared in a dairy herd. The presence of organisms in milk samples from affected animals was indicated by the appearance of yellow floccular colonies in incubated samples containing brom-cresol-purple (cf. Hotis test). Difficulty was experienced in isolating the causal organism. The only reliable method discovered was to incubate in closed vessels containing carbon dioxide (5–10 %). None of a large number of accessory growth factors, purine bases and crude extracts, incorporated in ox-blood agar, could replace gaseous CO<sub>2</sub>. Growth was very poor or absent in the usual liquid media except milk, and could be effected only by incubating in air containing added CO<sub>2</sub>, or by the addition of 10 % horse serum or of 1 % sodium bicarbonate if fermentable sugars were also present.

This organism was a streptococcus, producing typical zones of  $\beta$ -haemolysis in ox-blood agar. The majority of the strains tested gave a positive precipitin reaction with Group G serum only, the negative results obtained with some strains being probably due to poorness of growth in glucose broth. Most strains hydrolysed sodium hippurate, fermented aesculin and salicin, but not mannitol or raffinose, and produced an acid clot in milk.

Normal Group G streptococci, i.e. those growing in air, are not infrequently found infecting the bovine udder, but rarely seem to produce untoward symptoms nor are they contagious to any extent. The CO<sub>2</sub>-sensitive type, however, produced quite pronounced symptoms and appeared to spread very rapidly. The source of the organism is doubtful, as the infection must have been present for a considerable period before isolation was effected. This type of organism was isolated also from a single cow in another herd under the same management; at one period this animal had been infected with a normal-type Group G streptococcus which became CO<sub>2</sub>-sensitive after a dry period between lactations.

The infection was completely eradicated by the use of penicillin.

Thanks are due to the A.R.C. for a grant, part of which was used to cover the expenses of this investigation.

**Studies on Tick-Borne Fever in Sheep.** By ANGUS FOGGIE (*Gilmerton*)

The literature on tick-borne fever is reviewed. The observation that, although cattle show no symptoms, the infective agent of tick-borne fever may remain viable in their blood for up to 27 days after artificial infection, is confirmed. The infective agent is shown to be capable of survival in stored citrated whole blood for 18 days. Plasma from an infected sheep, filtered through a 1.5  $\mu$  gradicoid membrane, is shown to be infective, but the organism is retained by a 1  $\mu$  membrane.

The morphology of the causal organism is described and compared with that of *Rickettsia canis*, *R. bovis* and *R. ovina* (Donatien & Lestoquard, 1935–6). The tick-borne fever organism, although presenting comparable morphological characters, differs from these rickettsias in that it parasitizes the phagocytes and not the large lymphocytes.



The close similarity between the diseases caused by these rickettsias and tick-borne fever is noted. In all cases the incubation period is followed by an acute febrile attack and, on recovery, there is a prolonged period during which the parasite remains viable in the blood stream without causing further ill-health. Recurrence of the acute symptoms may be brought about by a disturbance of the balance between the reticulo-endothelial system and the parasite. Tick-borne fever has been shown to remain viable in a sheep for at least 20 months and an acute attack may be provoked by splenectomy of a latent case.

In the above rickettsial diseases and in tick-borne fever, it is difficult to produce a further attack of fever by reinfection while the animal is in the stage of premunition.

The name *Rickettsia phagocytophilia ovis* is proposed for the tick-borne fever parasite.

### **Factors Affecting the Organization of Submerged Cultures of Fungi.**

By R. B. DUCKWORTH and G. C. M. HARRIS (*Manchester*)

#### **A Method for Expressing Numerically the Morphological Features of Submerged Cultures of Fungi, with Special Reference to *Penicillium chrysogenum*, Q 176. By MURIEL BUTLER and G. C. M. HARRIS (*Manchester*)**

The method provides a rapid, routine means of comparing the morphology of large numbers of cultures of *Penicillium chrysogenum* grown under different conditions, and for recording observations in a convenient numerical form.

It is applied to those morphological features, such as degree of vacuolation or autolysis of the protoplasm, which cannot be measured directly. The results are expressed by means of a numerical scale ranging from 0 to 6 which is applied to each character; each number in the scale represents a certain stage in the expression of that character. Thus, in estimating the frequency of branching of the mycelium, a numerical result of 0 is given for those cultures in which no branching is observed, while the higher numbers of the scale are recorded for cultures having a much-branched mycelium.

Estimations are guided by sets of standard photographs, one set being prepared for each character. Each set consists of a series of photographs corresponding with the numbers of the scale. The morphological features of cultures are compared with each set of standards in turn and an appropriate number recorded for each character present in the culture.

This 'comparative' method of examination may be performed very rapidly. The maximum amount of time allowed for one culture at one stage of its growth is 20 min., but in practice it is found to be less since all the characters studied are seldom displayed by one culture. It has been found that the amount of variation in results obtained when the same cultures are examined by different observers or on different occasions is very small. In addition, results given in a numerical form are easily included in graphs or tables.

These advantages justify the use of the method in our present investigations and possibly its adaptation for similar work with other moulds.

**Bactericidal Action of some Quaternary Ammonium Compounds which contain more than one Paraffinic Chain.**

By J. G. DAVIS, J. C. L. RESUGGAN, ROBERTA IVE and H. M. WOOD (*London*)

Following the development of dioctyldimethylammonium bromide for a special purpose, and in view of the rather different properties and bactericidal power of that quaternary compound compared to previous single long chain types, a number of quaternary ammonium compounds have been prepared which all contain more than one hydrophobic chain independently attached to the nitrogen atom. Their bactericidal powers have been established, and it has been found that in the twin chain series beginning with dihexyldimethylammonium bromide and terminating with didodecyldimethylammonium bromide anti-bacterial activity increases with the length of the chains in a regular manner, which would be anticipated, whereas with long chain compounds bactericidal activity seems to terminate when there are 18 carbon atoms in the one hydrophobic chain. In the twin chain series the total of 24 carbon atoms still does not appear to have reached the limit. A dinonyl compound where the nonyl chains were not straight, but trimethylhexyl, was also prepared, and its activity found to be somewhere between the dihexyl compound and the diheptyl. This suggests, as indeed the authors had previously suspected, that it is not the total number of carbon atoms in a chain which is significant but rather the number of carbon atoms which may be recorded as being in a straight chain; thus branchings did not have very much effect.

It would appear that surface activity is influenced by the configuration of the hydrophobic chain in this way and must be considered an important factor in determining the anti-bacterial activity of compounds of this class. Most of the compounds have been tested at two or three pH levels against Gram-positive and Gram-negative organisms. They all show a regular tendency to higher anti-bacterial activity when the pH is in the neighbourhood of 10.5 to 11. In one or two cases where a more detailed study of the pH factor has been made a depreciation of activity seems to occur about pH 9.5, so that at that level activity is less than at 7.2 or at 10.4. This point will need confirmation for a variety of other compounds before any generalization can be ventured. Eleven compounds have been reported upon.

**The Immunological Relation between *Haemophilus pertussis* Toxic Filtrates and *Haemophilus pertussis* Extracted Toxin.**

By ANNIE M. BROWN (*Carshalton*)

A rabbit which had failed to respond to three courses of *H. pertussis* toxic filtrate by the production of detectable antitoxin in its serum, gave an immediate secondary response when injected with *H. pertussis* extracted toxin. Similarly, a rabbit given a primary stimulus of *H. pertussis* extracted toxin showed a secondary response when injected with *H. pertussis* toxic filtrate. The two necrotic toxins, if not identical, must be very closely related. Further evidence was obtained by a comparison of the titres of anti-toxic sera determined against both these toxins.

**The Detection and Isolation of a new Antibiotic from Culture Liquors of *Streptomyces griseus*. By M. LUMB (Nottingham)**

The production of antibiotics by *Streptomyces griseus* and many other biologically active organisms is a specific property of the strain and not of the species.

Streptomycin-producing strains of *Strep. griseus* are known to form at least four antibiotics, namely Streptomycin (Waksman, Schatz & Reilly, 1946), Streptomycin B (Fried & Titus, 1947), Actidione (Whiffen, Bohonos & Emerson, 1946), and an unnamed antibiotic isolated by Waksman *et al.* (1946), which appears to be contained to a greater extent than the other active products within the mycelium of the organism.

A possible further new and active substance inhibitory to several yeasts and fungi has been detected by the inhibition of strains of *Saccharomyces* in ditch plate tests by culture liquors of *Strep. griseus*. An activity not shown by Streptomycin or Streptothricin.

This new antibiotic is produced in nearly all media under the normal conditions employed for the production of Streptomycin. In synthetic media the yields of this substance are such that various species of *Saccharomyces* are inhibited by 1 in 250 dilutions of 8-day surface culture liquors. The activity appears to disappear in prolonged fermentations, whether these be by the surface or submerged processes.

The active substance differs from Streptomycin, Streptomycin B and Grisein in that it is soluble in common organic solvents and also in that it appears to possess no activity against *Staphylococcus aureus*, *B. subtilis*, *E. coli* or other tested bacteria. In these respects it closely resembles Actidione, with which it may be identical, although there does appear to be certain indications to the contrary. (Thus, for example, our substance appears to be completely innocuous while crude Actidione is said to be highly irritating to the skin.)

The yeast active substance can be readily extracted from culture liquors by (1) adsorption on charcoal, (2) elution with organic solvents of which acetone, methanol and butanol were found to be the most efficient and (3) by vacuum distillation of solvent at 45°C.

The crude substance at this stage is a brown solid (active against *Streptomyces logos* at a dilution of 1 in 250,000). This can be freed of associated pigments and further purified by butanol.

The charcoal remaining after the elution with organic solvent can be further eluted with acid methanol which will then yield normal recoveries of Streptomycin on subsequent treatment according to the Carter (1945) process.

The active substance appears to be relatively thermostable, but it appears to lose activity on prolonged storage even under refrigerator conditions at 4°C.

The full range of the compound's activity is not yet worked out but in addition to its activity against the yeasts it shows marked action against certain plant pathogens, including *Cryptococcus neoformans* and *Corticium solani*.

The compound appears relatively non-toxic, 40 g. mice tolerating a daily dose of 20 mg. of crude solid.

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## Susceptibility and Resistance to Staphylococcal Infection.

By IWO LOMINSKI (*Glasgow*)

Clotting of plasma by *Staphylococcus aureus* requires two factors, Staphylocoagulase and an 'activator'. The latter is present in rabbit and human plasma but deficient in the guinea-pig, mouse and fowl. Activator-deficient plasmas do not clot with staphylococci unless activator is added; activator-deficient animals are resistant to staphylococcal infection, but become sensitive when injected with clottable plasma (Smith & Hale, 1944, 1947; Smith, 1948). The resistance of certain species thus appears related to deficiency of activator.

The purpose of this study is to show that variations of resistance in man cannot be explained, like species resistance, by deficiency of activator. Another factor (Lominski & Roberts, 1946) called 'inhibitory substance', which prevents clotting of plasma by staphylococci, seems responsible for differences of resistance within the human species.

*Experiments.* By quantitative estimations of activator in sera it has been found that in most healthy individuals and patients suffering both from staphylococcal and non-staphylococcal conditions, the activator level was high; it is not correlated with staphylococcal infection.

In contrast with activator, the incidence of the inhibitory substance is significantly greater in the control population than in those suffering from a staphylococcal infection. This suggests that the inhibitory substance plays a part in man's resistance to staphylococci.

*In vivo* the action of the inhibitory substance was tested by giving one intraperitoneal injection to animals inoculated intravenously with virulent *Staphylococcus aureus* (three strains); Table 1 summarizes one experiment.

Table 1. *Forty rabbits*

| Human serum no. | Inhibitor titre | Anti- $\alpha$ titre | Death       | Survival |
|-----------------|-----------------|----------------------|-------------|----------|
| None            |                 |                      | 48-56 hr.   | None     |
| 1               | 1:8             | None                 | 153-177 hr. | None     |
| 2               | None            | 1:20                 | 48-53 hr.   | None     |
| 3               | 1:1000          | 1:10                 | None        | All      |
| 4               | 1:500           | None                 | None        | All      |

*Conclusions.* The 'inhibitory substance' can protect animals from a fatal staphylococcal infection or modify the course of the disease.

From previous work and our own it appears that staphylocoagulase is not antigenic; the inhibitory substance can thus not be considered as an antibody.

Accordingly, the inhibitory substance would represent a new category of resistance factors.

We are indebted to the Rankin Research Fund for a grant towards the expenses of this work.

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#### Some Factors Governing the Formation of Spores in *Bacillus anthracis*.

By J. W. HOWIE (*Aberdeen*)

An actively sporing anthrax culture formed spores in nutrient broth within 6 hr. at 37°C., as judged by development of heat resistance (60°C. × 80 min.) in the culture.

Spore-free bacillary suspensions were secured by passing this culture three times direct from mouse to mouse and emulsifying the spleen in distilled water. Bacillary suspensions readily formed spores under circumstances which permitted growth of the bacilli but they did not form spores if subjected to such unfavourable conditions as rapid drying or low temperature. In the ice-chest (0–4°C.) bacilli did not spore and died in 6 days. At room temperatures ranging from 7.2 to 17.2°C., bacilli in sterile milk failed to form spores and died between 4 and 6 days. If the milk was kept at 25°C. spores formed between 2 and 4 days. Spores were not found in mice that died or were killed up to 15 days after inoculation with spore-free bacilli.

Mice that survived inoculation with spore suspensions still had viable spores in their spleens for at least 8 months after inoculation.

#### Toxicity of Disintegrated *Bacillus anthracis*.

By H. K. KING and JEAN STEIN (*Edinburgh*)

*Bacillus anthracis* is one of the few highly pathogenic organisms in which, so far, there is no conclusive evidence of toxin production. The demonstration of an endotoxin would in any case prove difficult in view of the high temperature or drastic chemical treatment required to kill what is normally a sporing organism. In such cases, mechanical disintegration by shaking with minute glass particles (Curran & Evans, 1942; King & Alexander, 1948) has the advantage of killing spores as readily as vegetative cells and with minimal risk of damage to labile cell constituents. We have, however, employed this method with negative results.

*B. anthracis* (N.C.T.C. 5180: also our laboratory stock strain: average M.L.D. for 20 g. mouse, approx. 2000 organisms for both strains) was grown under conditions which it was hoped might simulate the natural conditions in the host, viz., on blood-broth, both aerobically, anaerobically, and semi-anaerobically, with and without the addition of 80% CO<sub>2</sub>. Ordinary media

(nutrient and meat-digest broth: also serum broth) were also tried. 20 hr. culture periods were used in most experiments, but periods of 6 hr. to 10 days were also used. Our experiments thus included both sporing and predominantly vegetative cultures. pH values from 6 to 8 were investigated, and incubation at 22° as well as 37°. In all cases washed suspensions were prepared and shaken with the glass particles (King & Alexander, 1948) for several hours, until the suspension was either completely sterile, or had too low a viable count to affect the result. Mice were then injected (usually intraperitoneally, sometimes subcutaneously or intravenously) with 300–400 million disintegrated organisms. In no case did this large dose produce death or even manifest toxic symptoms. It is possible that the organism produces toxin *in vivo* but not under artificial cultivation (cf. Cromartie, Bloom & Watson, 1947; Watson, Cromartie, Bloom, Heckley, McGhee & Weissmann, 1947). We infected mice with anthrax, and the spleens—containing vast numbers of the bacilli—were mechanically disintegrated. These disintegrates, too, were non-toxic when injected into mice.

The validity of this mechanical disintegration as a method of preparing endotoxins was tested by experiments with *Vibrio cholerae*. Here we found that the lethal dose was of the same order for disintegrated as for heat-killed organisms.

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#### The Virulence-Enhancement Factor of Gastric Mucin.

By H. K. KING and A. F. MACCABE (*Edinburgh*)

Castles & Miller (1936) demonstrated that infection could be established by intraperitoneal injection (with certain bacteria) with a smaller number of organisms if the latter were suspended in a solution of mucin instead of some inert fluid such as saline. Gould & King (1947) demonstrated the possibility of obtaining from commercial hog gastric mucin ('Granular Mucin Type 1701-W', Wilson and Co., Chicago) a fraction in which this virulence-enhancement factor was concentrated. Their method, involving a differential centrifugation technique, has proved difficult to repeat, but we have obtained more consistent results by the following method.

An aqueous suspension of the mucin was adjusted to pH 7–7.5 and allowed to stand at 0° overnight. It was then centrifuged at high speed (1–2 hr. at R.C.F. 20,000 × g., using a Blitz or Sharples centrifuge). The viscous supernatant was discarded and the solid particulate matter dried and then treated with formamide. After allowing several hours for the material to disperse in the solvent, the insoluble matter was centrifuged down (15 min. on an ordinary centrifuge—R.C.F. 2000 × g.) and discarded. The formamide solution was dialysed against water to remove the formamide and the remaining suspension freeze-dried.

This product represented 4 % of the original mucin and exhibited about twelve times its virulence-enhancing potency. It dissolved in water at slightly alkaline pH to give an almost non-viscous solution. It is not identical with the human blood-group A factor known to be present in hog gastric mucin (Morgan & King, 1948): this remains in the first viscous supernatant. It retained, after prolonged dialysis, 11 % of mineral matter, and contains 7.7 % N (Kjeldahl). It is probably still not homogeneous and therefore no detailed chemical investigation has been undertaken. The Molisch and Sakaguchi (arginine) reactions were strongly positive, the biuret and Millon reactions positive, the tryptophane reaction weak.

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#### **Molybdenum and Formic Dehydrogenase of Bacteria.**

By JANE PINSENT (*London*)

It was found that when coliform bacteria were grown on an inorganic medium containing nitrate, from which trace-metals had been removed, that no formic dehydrogenase was produced unless molybdenum was added to the growth medium.

The medium containing glucose, lactate, nitrate, ammonia and phosphate was purified by extracting with 8-OH quinoline in chloroform solution; all glassware was Pyrex and carefully cleaned (Waring & Werkman, 1942). Mg and Fe were added to the purified medium in concentrations of 5 and 0.1  $\mu\text{g./ml.}$  respectively, and Cu, Co, Mn and Zn in concentrations of 0.01  $\mu\text{g./ml.}$  Growth was not affected by the omission of molybdenum, but the final pH of the medium was in general lower if no molybdenum was present.

The cells were harvested and washed, and formic dehydrogenase activity estimated manometrically by oxygen uptake with formate as substrate, and by reduction of methylene blue in Thunberg tubes. The  $Q_{O_2}$  of deficient cells was usually 10 or less, while that of cells grown in the presence of molybdenum was 70–100. No effect on  $Q_{O_2}$  was observed on adding molybdenum to washed suspensions of deficient cells.

In the medium used the yield of cells obtained was of the order of 200 mg./l. Under these conditions, a maximum effect was obtained by adding molybdenum as ammonium molybdate to a final concentration of  $10^{-8}$  g. atom Mo/l. A detectable effect was produced at  $10^{-10}$  g. atom/l. The effect was specific, and molybdenum was not replaceable by vanadium, selenium, chromium, tungsten or uranium.

Molybdenum does not appear to be necessary for the production of formic dehydrogenase if nitrate is omitted from the growth medium.

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## Some Aspects of Sulphur Metabolism in Soils.

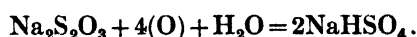
By H. GLEEN (*Long Ashton, Bristol*)

Using a soil perfusion technique (Audus) it has been possible to study the dynamic bio-chemical changes of certain sulphur compounds in soil. (Gleen & Quastel, unpublished.)

The metabolism of thiosulphates and other polythionates in soil is complex and is influenced by the specific conditions prevailing in the soil environment. Thiosulphate undergoes two types of biological oxidation in soil: (1) to tetrathionate and sulphate, the tetrathionate being further oxidized to sulphate, (2) to sulphur and sulphate.

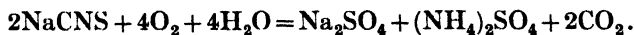
The tetrathionate can be biologically reduced to thiosulphate and a cyclic process can be established.

Bacteria-enriched soils oxidizing thiosulphate may be obtained by perfusion of thiosulphate alone and may be studied as a specific enzyme system: using a new Warburg technique respiration studies of these soils may be obtained and it has been shown that the oxygen uptake required to metabolize a known quantity of thiosulphate conforms to the formula



Tetrathionate and trithionate are metabolized by the same enzyme system, but not dithionates or sulphur, and oxidation seems to depend on the activation of a polythionase system due to the presence of unsaturated sulphur groupings in these sulphur compounds.

Thiocyanate is also metabolized in soil by another specific microflora (Gleen, unpublished) and is oxidized to ammonium sulphate and carbon dioxide as follows:



Soils can be enriched by thiocyanate perfusion, and these will also metabolize thiosulphate and tetrathionate but not dithionate, which suggests that a similar enzyme system is present in these organisms as in the thiosulphate oxidizing organisms. The reverse is not found. A thiosulphate-enriched soil will not metabolize thiocyanate, probably due to the toxic action on these bacteria.

Adaptation of the thiocyanate organisms to concentration of thiocyanate can be shown. The rate of oxidation of thiocyanate increases to a maximum at  $m/25$  approx., diminishing to zero at  $m/5$  thiocyanate.

$\text{O}_2$  uptake of these thiocyanate-enriched soils has also been studied, and it has been demonstrated that the enzyme system responsible for this oxidation is a constitutive one.

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**The Action of Micro-organisms on Pyridine.** By F. W. MOORE (*Birmingham*)

When pyridine solutions are added to the soil, the following facts have been established: (1) the pyridine disappears (Buddin, 1914); (2) there is a marked increase in the numbers of bacteria present (Robbins, 1917); (3) the nitrogen of the pyridine ring is rapidly made available for plants (Funchess, 1917).



Pyridine can act as sole source of nitrogen for a number of micro-organisms, including laboratory strains of *Aerobacter aerogenes* and *Serratia marcescens* (den Dooren de Jong, 1926). Growth of pure cultures of *Proactinomyces* is possible in a medium containing pyridine as sole carbon and nitrogen source (Moore, 1948).

The carbon:nitrogen ratio of pyridine is approximately 4:1; and as the organism must obtain its energy by the oxidation of part of the pyridine, it is probable that the ratio of the amounts of carbon and nitrogen available for assimilation will be considerably less than 4:1. Thus it is unlikely that the organism can assimilate all the nitrogen present. Tests for the presence of inorganic nitrogen compounds in culture filtrates were all negative except in the case of ammonia.

The ammonia content of culture filtrates from media containing 0.05–0.80 % pyridine were estimated and recorded graphically. The resulting curves showed, that in the case of 0.05 % pyridine, the ammonia content reached a maximum after 20 days and that the ammonia released corresponded to 87 % of the total nitrogen added to the medium in the form of pyridine.

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#### Group Relationships between Bacteria Shown by Amino-acid Metabolism.

By H. PROOM and A. WOIWOD (*Beckenham*)

The amino-acid and polypeptide composition of bacterial filtrates from cultures of 250 strains, representing eighteen different genera, has been examined by single dimensional partition paper chromatography using the technique previously described (Woiwod & Proom, 1948). The cultures were examined after growth for 3 days at 37°C., during which period the pH remained slightly alkaline, and also after being held for a further 7 days at both slightly alkaline (pH 7.6–8.5) and acid (pH 4.5) reaction. Some of the effects observed are reported here.

(a) *The removal of amino-acids during the first 3 days of growth.* When the chromatographic picture of the filtrate is affected by the growth of any organism, the serine spot is always the first spot to decrease in intensity. Serine would therefore appear to be an amino-acid of particular significance in bacterial metabolism. With Gram-negative organisms the aspartic acid spot is not affected, but with most Gram-positive organisms the aspartic acid is rapidly removed. The medium used contains appreciable amounts of ammonium salts, and with those organisms which grow on media containing inorganic nitrogen as the sole source of nitrogen, there is practically no change in the chromatogram during the initial growth stage.

(b) *The order of the disappearance of amino-acids during the 10-day period.* Bacterial growth is practically complete during the first 8 days, but when the cultures are examined after a further 7 days other extensive changes in the chromatogram may be observed. The chromatographic picture suggests that these changes occur in a well-defined order which may be characteristic of the species or group. With *Proteus* at either acid or alkaline reaction the order of disappearance of the amino-acids as they occur on the chromatogram is serine, leucine group, valine group, glutamic acid, alanine, the basic amino-acid group and finally aspartic acid, leaving two polypeptide spots. With *E. coli* at acid reaction serine, the basic amino-acid group, glutamic acid and alanine disappear, followed by the appearance of a polypeptide near the tyrosine position on the chromatogram. Finally, the valine group and leucine group, followed by the polypeptide and aspartic acid, disappear.

(c) *The appearance of species- or group-specific polypeptides.* Many species synthesize extracellular polypeptides which are species or group specific. The following are examples: *Proteus*, *Staphylococcus*, *Cl. tetani*, *Cl. bifermentans* at alkaline reaction and *Cl. perfringens*, *E. coli* and *Shigella* at acid reaction. These polypeptides always appear concurrently with other changes on the chromatogram; the tetanus polypeptide with the disappearance of glutamic acid, the bifermentans polypeptide with the disappearance of proline and the staphylococcus polypeptide with the disappearance of alanine, and so on. The structure of these polypeptides has not yet been examined, but in the case of *Cl. tetani* the polypeptide spot was eluted from the paper and hydrolyzed. In spite of the fact that a slight reduction in serine and a marked reduction in glutamic acid were the only other changes noted on the chromatogram, the polypeptide hydrolysate contained most of the known amino-acids and the polypeptide therefore was not a polymer of glutamic acid, as might have been expected.

Although it is too early to state the form it will take, it is evident that it will be possible to make a reasonable classification of bacteria based on their amino-acid metabolism as shown by paper chromatography. This should be of considerable value in determinative bacteriology, since it is probable that amino-acid and protein metabolism have more phylogenetic significance than the physiological reactions concerning carbohydrate metabolism which are in more common use.

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#### Microbial Breakdown of Structural Starches in the Digestive Tract.

By F. BAKER, H. NASR and F. MORRICE (*Aberdeen*)

Direct microscopy has been employed to compare the relative importance of microbial agents and digestive secretions in the breakdown of raw maize and raw potato starches during passage through the digestive tract of rats, mice, hamsters, rabbits, guinea-pigs, domestic pigs and oxen. Disintegration of the

starch granules was determined by noting loss of birefringence in polarized light and alteration of histochemical reactions to iodine and Congo red. In rats, mice, rabbits, guinea-pigs and pigs, maize starch was broken down by digestive secretions in the small gut. Bacterial action was inconspicuous and few granules reached the caecum. Potato starch, on the contrary, mostly escaped digestion in the small gut and large numbers of granules were observed in the caecum in process of disintegration by bacteria. In mice, tuber starches such as canna, tapioca and maranta arrowroot showed a similar picture to that with potato starch. Amongst cereal starches, rice and wheat showed a similar picture to maize. In ruminants both maize and potato starch were attacked in the rumen, the maize more rapidly than the potato. In the hamster bacterial disintegration of maize and potato starches was observed in the stomach diverticulum as well as in the caecum. Microbial breakdown was throughout associated with a marked rise in density of the Gram-positive and iodophile microflora. The bacteria immediately concerned in disintegration exercised their action *in situ* upon the surface of the granules. Structural factors influenced the mode of breakdown and dietary value of potato starch for rats fed a diet lacking vitamin B. Through the kindness of Drs L. H. Lampitt and D. B. Hughes of Messrs Lyons' Laboratories we were able to study potato starches ground in a ball mill to 81, 86 and 98 % solubility in cold water and to compare the picture with unground starch. Unground starch was associated with light-coloured bulky faeces and refection: that is to say, the rats gained weight and remained well although their diet lacked vitamin B. But if the starch was ground to 98 or 86 % solubility most of it was digested in the small gut, the faeces were dark and the rats lost weight and showed symptoms of avitaminosis, including acrodynia and polyneuritis, which culminated in death of the animals.

These observations led to cultural and other investigations which are still in progress. A large iodophile butyric-acid producing clostridium, directly associated with the disintegration of granules in the caecum of pigs fed potato-starch diets, was isolated in pure culture. This organism broke down structural as well as soluble starches *in vitro*. Cell-free filtrates of fluid cultures showed powerful amylase activity. The rate of growth and amylase activity were greatly accelerated by addition of biotin and *p*-aminobenzoic acid to certain of the media used for culture of the organism. A preliminary microbiological assay demonstrated that the organism was capable of synthesizing riboflavin and nicotinic acid.

Grateful acknowledgements are due to Drs M. McNaught and E. C. Owen of the Hannah Dairy Research Institute, Kirkhill, Ayr, for undertaking for us the feeding and other experiments with oxen.

#### **'Trace Elements' in Rumen Micro-organisms.**

By R. L. MITCHELL and J. TOSIC (*Aberdeen*)

It is known that certain 'trace elements' are essential factors in animal nutrition, for if they are in short supply in the diet deficiency diseases may result (for reviews see: Maynard & Smith, 1947; Russell, 1944). The mechanism of

action of such small quantities of trace elements is still either unknown or obscure, but they have been invariably associated with the direct requirements of the animal. In a recent study of cobalt concentration in different fractions separated from the rumen contents of sheep, it has been for the first time demonstrated (Tosic & Mitchell, 1948) that: (a) micro-organisms of the rumen accumulate cobalt from their external environment; (b) the concentration of cobalt in the microbial population of the rumen is related to the concentration of cobalt in the diet; and (c) a large proportion of cobalt present in the rumen contents is situated in the micro-organisms, the concentration in the external environment being very low. This work has now been extended to copper, nickel, molybdenum, zinc, vanadium, titanium, manganese and iron.

Table 1. *Contents of Cu, Ni, Mo, Zn, V, Ti, Mn, and Fe in microbial and other fractions separated from rumen contents of sheep gaining weight on adequate hay diet*

| Fraction of rumen contents | P.p.m. in dry matter |       |      |     |      |      |     |     |
|----------------------------|----------------------|-------|------|-----|------|------|-----|-----|
|                            | Cu                   | Ni    | Mo   | Zn  | V    | Ti   | Mn  | Fe  |
| Microbial (I)              | 40.5                 | 10.35 | 1.97 | 136 | 2.00 | 87.0 | 393 | 783 |
| Microbial (II)             | 39.0                 | 14.60 | 5.38 | 220 | 2.10 | 53.1 | 252 | 992 |
| Microbial (III)            | 72.0                 | 8.72  | 3.27 | 186 | 0.90 | 24.7 | 242 | 535 |
| Plant particles (IV)       | 11.5                 | 1.98  | 0.23 | 34  | 0.28 | 10.7 | 118 | 113 |
| Supernatant (V)            | 31.2                 | 2.66  | 0.67 | 12  | 0.43 | 8.4  | 94  | 30  |
| Hay as fed                 | 6.3                  | 1.11  | 0.48 | 35  | 0.72 | 30.6 | 150 | 209 |

From our findings, summarized in Table 1, it is seen that Cu, Ni, Mo, and Zn are some ten times more concentrated in micro-organisms of the rumen (in an adequately fed sheep) than in the food on which this animal was maintained; in case of V, Ti, Mn and Fe the extent of accumulation is less pronounced. The rumen liquid from which fractions I-IV were separated still retained some suspended micro-organisms, and figures for the supernatant (V) for some of the reported 'trace elements', are probably mainly due to them. Taking into account the contribution of each of the separated fractions towards the dry matter of the unfractionated rumen contents, we find that a large proportion of Ni, Mo and Zn, and to a lesser degree of V, Ti, Mn, and Fe of rumen contents is situated in micro-organisms of the rumen. Although it is known (Waring & Werkman, 1942, 1944) that iron is a necessary growth factor for *Aerobacter indologenes*, and that Fe deficiency suppresses certain enzymic activities of this organism, there is little published information on the requirements and role in bacterial metabolism of other elements reported in this communication.

The accumulation of certain 'trace elements' in micro-organisms of the rumen and some organs of the host, and their distribution between micro-organisms of the alimentary canal and their external environment may assume some importance in aetiology of diseases due to a deficiency of certain 'trace elements'.

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**The Bacterial Surface. Entry of Amino-acids.**By K. MCQUILLEN and P. D. MITCHELL (*Cambridge*)

In a series of studies which have appeared in volume I of *The Journal of General Microbiology*, it has been shown by Gale and his collaborators that in growing cultures of Gram-positive organisms, lysine and glutamic acid of the medium are collected by the cells and concentrated internally, apparently in the free state. Lysine becomes concentrated in resting cells spontaneously, while the collection of glutamic acid requires the metabolism of some energy yielding substrate. Since this ability appears to be peculiar to Gram-positive organisms, we thought it of interest to attempt to obtain more information about its mechanism.

The studies described in this paper were made on *Strep. fecalis* (Lancefield group D) by electrophoresis, using a cylindrical cell and determining the electrophoretic velocity microscopically in the stationary layer under a known potential gradient.

When suspended in solutions of ionic strength 0.005 or greater, the mobility of cells containing little lysine or glutamic acid is not changed after amino-acid accumulation has occurred. This shows that little or no amino-acid is adsorbed at the external surfaces of the cells.

At ionic strengths below 0.005, the presence of lysine in the cells causes a decrease in mobility (a reduction of the net negative charge) which becomes more pronounced as the ionic strength is lowered.

Theoretical considerations by Mitchell, to be published in due course, indicate that at very low ionic strengths, when the double electrical layer becomes very thick, the material underlying the surface of the bacterial cell contributes to the net charge. In the light of this, it seems significant that the change in the net charge brought about by the lysine becomes appreciable when the thickness of the double layer is of the same order as that of the external envelope of the cells. Electron microscope and direct gravimetric determinations agree in estimating the envelope thickness as about 100 Å.

These observations are equally compatible with two mechanisms for the collection and retention of lysine by streptococci. Either the lysine may enter under a potential gradient and become uniformly distributed inside some structure about 100 Å. below the external surface of the cells, or it may be retained by adsorption. But in the latter case, the part of the lysine which contributes to the change in net charge must be situated about 100 Å. from the cell surface, that is, in the vicinity of what is normally accepted as the plasma membrane.

**Estimation of Growth Rate of a *Staphylococcus*. By P. D. MITCHELL and T. VALYI-NAGY (*Cambridge*)**

For purposes beyond the scope of this paper, we were interested in estimating the rate of growth of *Staph. aureus* Duncan rather more accurately than is possible by the usual methods.

The factors determining reproducibility and absolute accuracy fall naturally into three groups associated with conditions of growth, technique of sampling and method of estimation of growth.

The organisms were grown on a casein hydrolysate glucose medium buffered with phosphate at pH 6.5; and a very simple technique was developed for maintaining standard conditions of stirring and aeration in the cultures during growth.

Samples were withdrawn from the culture with sterile pipettes and growth stopped by cooling the samples to 0°C. or mixing with a small volume of formaldehyde solution to give a final concentration of 1 %.

The determination of the growth was made by direct gravimetric estimation of dried organisms for values above 500  $\gamma$ /ml., and with the Beckmann photo-electric spectrophotometer for values down to 40  $\gamma$ /ml.

When the dry weight is determined in the spectrophotometer, for wavelengths in the blue and ultra-violet, in addition to the light scattering by the cell suspension, there is an appreciable absorption by the constituents of the cells. The strongest absorption at 260  $m\mu$  would be accounted for by 10 % dry weight of nucleic acid, while a subsidiary absorption at 280  $m\mu$  is presumably due to the proteins of the cells. When the light scattering is reduced by suspending the cells in glycerol, an absorption curve very similar to that of a nucleoprotein is obtained. The possible value of such measurements is obvious.

The sensitivity of the photometric measurements of growth is greater in the blue and ultraviolet than in the red; but the relationship between the photometer reading and dry weight is subject to more complex changes in the region where constituents of the cells absorb strongly than in the region where scattering is predominant. Accordingly, most of our measurements were made at 850  $m\mu$ .

As our medium absorbed in this region, it was necessary to centrifuge samples during growth to use as controls. Precautions were necessary in the sampling technique to avoid small dilution errors since these would cause large photometric errors.

Over the range of our estimations, the mean deviation of the determinations was approximately 1 %. The growth curves from the latter part of the lag phase to close to the maximum stationary phase were reproducible to within the limits of error of the methods of sampling and estimation.

The rate of growth is a function, not of the age of the culture, but of the bacterial population density. It follows that for growth on a given medium, the cell population density is often a better index of the physiological condition of the cells than the age of the culture.

#### **Electron Microscope Studies of the Adsorption of Certain Viruses on Fowl Red Cell Membranes. By I. M. DAWSON and W. J. ELFORD (London)**

Carefully prepared suspensions of laked fowl red cells adsorb viruses of the influenza, Newcastle disease, fowl plague and mumps group as well as do the intact cells, under appropriate conditions. We have been able to mount and

shadow with palladium for examination in the electron microscope such as laked cells following contact with virus suspensions. Most striking photographs of the above-mentioned viruses adsorbed to the cell membranes have been obtained. There appears to be no regular pattern of adsorption, the particles being distributed randomly, with occasional tendency to chain formation. Adsorption is quite selective and excellent fields are obtained after shaking the 'ghosts' with undiluted allantoic fluid. Furthermore, the degree of adsorption indicated by direct counts of the particles per given area of membrane provides a basis for the roughly quantitative interpretation of some virus-cell relationships. The implications of our experimental results to date will be discussed.

## DEMONSTRATIONS

MURIEL BUTLER and G. C. M. HARRIS (*Manchester*). 'A method for expressing numerically the morphological features of submerged cultures of fungi, with special reference to *Penicillium chrysogenum*.'

MARY E. ADAMS, K. R. BUTLIN and MARGARET THOMAS (*Teddington*). 'The morphology of sulphate-reducing bacteria.'

A. FOGGIE (*Gilmerton*). 'Tick-borne fever bodies in leucocytes.'

D. SLAVIN (*Gilmerton*). 'The sterilization of large quantities of broth by filtration.'

D. SLAVIN (*Gilmerton*). 'The intermittent aeration of broth cultures during incubation.'

J. CRUICKSHANK (*Aberdeen*). 'Bacteria whose colonies are migratory on solid media.'

J. CRUICKSHANK (*Aberdeen*). 'A collection of luminous organisms from North Sea fish.'

J. CRUICKSHANK (*Aberdeen*). 'Demonstration of "sugar" reactions on nutrient agar plates by the use of strong solutions of sugars (10-50 %) and the Liesegang rings which are produced.'

J. CRUICKSHANK (*Aberdeen*). 'The production of bacterial cellulose and the properties of the "Paper" so formed, with a mention of its use in chemical research.'

F. BAKER, H. NASR and F. MORRICE (*Aberdeen*). 'Microbial breakdown of structural starches in the digestive tract.'

P. D. MITCHELL (*Cambridge*). 'A simple device for aeration and stirring of cultures.'

G. B. LUDLAM (*Edinburgh*). 'A selective medium for isolating *Staph. aureus* from heavily contaminated material.'

HELEN ROSS (*Beckenham*). 'Morphological variations shown by *Cl. welchii* type B prior to spore formation.'

[*The Editors of the Journal of General Microbiology accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.*]

## THE SOCIETY FOR GENERAL MICROBIOLOGY

*The Eighth General Meeting of the Society was held in the Royal Institution, Albemarle Street, London, W.1 on Wednesday and Thursday, 20 and 21 April 1949. On the 20th there was a Symposium on 'The Nature of the Bacterial Surface', to which the following contributions were made:*

### COMMUNICATIONS

**Introduction to the Symposium.** By N. W. PIRIE (*Rothamsted*), *Chairman*

**The Surface Structure of *Shigella shigae* as revealed by Antigenic Analysis.**  
By W. T. J. MORGAN (*London*)

**The Nature of the Surface of Gram-Positive Bacteria.** By M. STACEY (*Birmingham*)

**The Osmotic Barrier in Bacteria.** By P. MITCHELL (*Cambridge*)

**On the Mechanism of Adsorption of Bacteriophages on Host Cells.** By T. F. ANDERSON (*Philadelphia*)

**The Status of Some Arguments about the Bacterial Surface.** By A. A. MILES (*Hampstead*), *Chairman*

**The Nature of Bacterial Surfaces.** By E. T. C. SPOONER (*London*)

**Capsule Formation in the Pneumococcus.** By HARRIETT E. TAYLOR (*Paris*)

**Bacterial Surface, Flagella and Motility.** By A. PIJPER (*Pretoria*)

*On 21 April the following Original Papers and Demonstrations were presented:*

**Some Factors Affecting the Multiplication of Coliphage.** By D. E. DOLBY and J. W. CZEKALOWSKI (*Leeds*)

**Enzyme Inhibitors and the Synthesis of Coliphage.** By J. W. CZEKALOWSKI and D. E. DOLBY (*Leeds*)

**Filamentous Structure Associated with Newly Isolated Strains of Influenza Virus.** By C. M. CHU, I. M. DAWSON and W. J. ELFORD (*Hampstead*)

**Growth Inhibitory Effect on *Shigella dysenteriae* which Occurs with Some Batches of Nutrient Agar and is Associated with the Production of Peroxide.** By H. PROOM, A. J. WOIWOD, J. M. BARNES and W. G. ORBELL (*Beckenham*)

Papain digest broths containing 7.5 g. TN/l. and 3.5 g. TN/l. were subjected to a number of tests using *Sh. dysenteriae*, strain CN 191, as the test organism. It was found that, with a moderate inoculum, the total growth after 24 hr. incubation was greater with the strong broth. The smallest inoculum to grow



was the same with both broths. When the broths were converted into nutrient agars the total amount of growth was the same when a heavy inoculum was used. However, the number of colonies which appeared, after plating with dilute inocula and subsequent incubation, were from 10 to 100 times greater with the agar made from the weak broth.

When the agar made from the strong broth was progressively diluted with saline agar, the number of colonies which appeared, after inoculation with dilute inoculum, increased and continued to increase until the medium was no longer capable of supporting growth. When silica gel was substituted for agar the phenomenon could not be reproduced. There was apparently no difference between diluted and undiluted broths.

Evidently from these experiments a growth factor was not involved. The phenomenon was not due to an inhibitor present in the agar, since dilution with saline agar increased the number of colonies which grew. It was not due to an inhibitor in the broth, since the smallest inoculum to grow was the same in both broths.

*Sh. dysenteriae* was then included as a test organism for the routine bacteriological testing of batches of nutrient agar. The test consisted of placing dilutions of an overnight broth culture of the organism on agar plates using the Miles and Misra (1938) technique.

It was noted that there was considerable batch variation. A 'good' nutrient agar would give confluent growth with an inoculum dilution of  $10^{-4}$ , numerous colonies with  $10^{-5}$  and about 20 colonies with  $10^{-6}$ . A 'bad' agar would give confluent growth with  $10^{-1}$  and a sharp cut-out with no growth at  $10^{-2}$ . A 'good' agar could be turned into a 'bad' agar by autoclaving several times. The 'good' and 'bad' agars were indistinguishable when tested with *Staph. aureus* or *Sh. paradysenteriae*. It was remembered that both these organisms, in contradistinction to *Sh. dysenteriae*, produce catalase. The inhibitory effect present in 'bad' agar was removed by dilution with saline agar.

Using a spot technique it was found that a number of substances such as  $\text{MnO}_2$ , some vegetable extracts, blood or purified catalase would neutralize the inhibition. A 'bad' agar could be turned into a 'good' agar by the addition of 1 g./l. of  $\text{MnO}_2$ . In the presence of this quantity of  $\text{MnO}_2$  it could be re-autoclaved and would remain satisfactory. After filtration to remove  $\text{MnO}_2$  it was still satisfactory, but on re-autoclaving it again became inhibitory.

From these results it seems obvious that some broths heated in the presence of agar produce an inhibitory substance which is almost certainly a peroxide. The removal of the peroxide reverses the inhibitory effect but does not influence the capacity of the broth to produce further peroxide on re-heating. No information is available as to the nature of the substances involved or the reasons for batch variation. Reducing the heat to a minimum during the preparation of both the broth and the agar reduced the number of bad batches produced.

**The Formation of CO<sub>2</sub> and Acetoin from Pyruvate by *Lactobacillus plantarum*.** By ELIZABETH ROWATT (*Sheffield*)

Washed suspensions of *Lactobacillus plantarum* (N.C.T.C. 7220) decompose pyruvate to give acetoin and CO<sub>2</sub>, with a  $Q_{CO_2}$  at pH 4 and 37° of 150-300. The rate of CO<sub>2</sub> evolution under these conditions slows down after 10-30 min. incubation with substrate. At higher pH, the rate of reaction falls more slowly, showing that the low pH accelerates inactivation. Glucose increases the initial  $Q_{CO_2}$  and delays the fall in activity. If cells are incubated at pH 4 at 37° without substrate and then incubated with pyruvate, very little CO<sub>2</sub> or acetoin is found. Glucose and a boiled extract of yeast restore much of the activity when added together, but not when added separately. The reaction is restored to a lesser extent by manganese and magnesium salts with glucose.

**Staining of Flagella.** By A. VOUREKA and A. FLEMING (*Paddington*)

The object of this communication is to bring out two points:

(1) A method of growing bacteria in slide culture for examination by phase contrast and subsequent staining. The bacteria are spread on a cover-slip and allowed to dry. Then two or three drops of nutrient agar (or agar containing penicillin) at a temperature of 48-50° are dropped on the cover-slip. As soon as this has set the agar covered slip is inverted on a microscope slide and is then ringed with paraffin or put in a moist chamber to prevent drying. After a suitable incubation at 37° or room temperature, the culture was examined by phase contrast without any disturbance, and if it appeared in the right stage the cover-slip was removed with forceps when it invariably carried with it the agar. We then had a cover-slip with a culture between it and a layer of agar about 1 mm. in thickness. This could be placed in a bath of formalin and the culture fixed *in situ*.

(2) After fixation for 2 days in formalin (undiluted) it was found that when flagella had been seen in the living culture by phase contrast they could be stained without mordanting by many simple stains. The best we found to be Night Blue (Gurr), a saturated watery solution. Time of staining 5 min.

**The Effect of Penicillin on Morphology.** By W. H. HUGHES, I. R. H. KRAMER, and A. FLEMING (*Paddington*)

It is well known that sublethal doses of penicillin in the culture medium produce morphological changes in many bacteria. The phase-contrast microscope allows these changes to be observed very easily when bacteria are grown on agar containing penicillin in miniature 'slide culture' which can be observed without disturbance. Most of our work was done with *Proteus vulgaris*. The changes observed vary with (1) temperature, (2) concentration of penicillin, (3) presence of free fluid between agar and cover-slip.

The changes seen are:

(1) Enormous elongation. These rods, which are perhaps five times thicker than a normal bacillus, may be anything up to  $200\mu$  in length.

(2) Productions of single or multiple swellings on the rods. These may be up to  $20\mu$  in diameter and of irregular outline when the agar is tight against the cover-slip, but are spherical and smaller when there is free fluid. Often completely spherical forms of  $6\mu$  or less are seen actively motile.

(8) Branching. This is common when there is a small amount of free fluid under the cover-slip and the concentration of penicillin is 10 units/ml.

The changes happen most rapidly at  $37^{\circ}$  and with higher concentrations of penicillin. The enormous elongation and branching occur best when the culture is grown at room temperature ( $18^{\circ}$  or less).

The movements of the mis-shapen organisms are fantastic. With the very long forms there is a tendency to form whorls which rotate for hours in the same microscopic field.

Similar but less marked changes are seen with *B. typhosus* and *B. coli*, also with *V. cholerae*. With this last organism spiral forms are produced indicating that growth has proceeded up to the point of the new organism acquiring a curved form but there is no separation of the individual elements. Enormously long twisted forms are produced.

In these abnormal forms nuclear material could, by appropriate staining, be demonstrated at intervals along the threads and often there seemed to be a concentration in the bulbous swellings.

On transfer to medium containing penicillinase these abnormal forms divide into normal bacteria.

#### **The Advisory Bacteriological Nomenclature Committee: its Aims and Objects.** By A. A. MILES (*Hampstead*) and S. T. COWAN (*Elstree*)

The terms of reference of the committee include drawing up a bacteriological nomenclature acceptable to most British workers, and forming opinions on problems to be discussed by the International Nomenclature Committee and its Judicial Commission.

Nomenclature must be based on a systematic classification; subcommittees have been set up to make detailed recommendations on the definitions of genera and the recognition of species within them. The committee intends to work out a classification that is historically less comprehensive than Bergey's, a system from which inadequately described species will be excluded; in short, a practical scheme in which controversy can be resolved by reference to strains actually in existence. Appropriate names for the genera and species are governed by the Bacteriological Code which recognizes as valid any name published since 1758 if it conforms to certain rules. This date is open to objection and the committee can, if it thinks fit, recommend to the International Committee that a later date be substituted.

**A Comparative Study of the Nutrition Requirements of the Genus *Bacillus*.**By B. C. J. G. KNIGHT and H. PROOM (*Beckenham*)

This is a preliminary report of a comparative survey of the exact nutritional requirements of species in the genus *Bacillus*. The object has been, in the first place, to see to what extent exact nutritional characters might be species specific. A longer-term objective is to accumulate material which might contribute to the study of taxonomic and evolutionary problems.

Typc-specimens of various species and numbers of cultures freshly isolated from natural sources have been examined. All cultures of most of the species examined have grown on relatively simple, defined media which contained glucose as main energy source.

For example, various specific media contained: (a) ammonia (*B. subtilis*, *B. megatherium*); (b) several specific amino-acids (*B. cereus*); (c) ammonia + biotin (*B. polymyxa*, *B. pumilus*); (d) ammonia + biotin + aneurin (*B. macerans*). Besides specific amino-acids only biotin, aneurin, or biotin + aneurin, have been the essential metabolites required as nutrients. With very few exceptions all the freshly isolated cultures of any one of the species so far examined have shown the same typical nutritional picture.

**The Utilization of Starch by Virulent and Avirulent Strains of *Haemophilus pertussis*.** By A. M. JAMES (*Greenford*)

Virulent strains of *H. pertussis* require starch for growth in a fluid medium; avirulent strains grow readily in its absence. The starch can be replaced by charcoal or by amylose, but not by any other carbohydrate, without considerably reducing the growth. The starch may be acting as a source of carbohydrate, as an adsorbent of toxic products, or may possibly combine the two functions.

Growth of virulent strains of *H. pertussis* in the fluid medium for 12 days causes a 20 % decrease in the starch concentration, but there is no decrease in starch concentration when avirulent strains are grown in the medium for 12 days.

Suspensions of virulent organisms (harvested from Bordet-Gengou medium or fluid medium) in a phosphate buffer containing starch cause a breakdown of starch to reducing sugars equivalent in amount to 20 % of the starch originally present. Suspensions of avirulent organisms show little, if any, breakdown of starch even after 5-6 days' incubation. Similarly, amylose is degraded by suspensions of virulent but not of avirulent organisms.

**Factors Affecting the Agglutinability of Red Cells by *Haemophilus pertussis*.** By J. UNGAR and A. M. JAMES (*Greenford*)

Suspensions of virulent strains of *H. pertussis* agglutinate human and laboratory animal (particularly rat and chicken) red cells; avirulent strains do not. The reading of the agglutination is made on the same principle as reading the

agglutination of blood groups A-B; recording the influenza virus agglutination pattern gave inconclusive results. We have found the optimal temperature for agglutination to be between 42 and 46°. We have confirmed the findings of Keogh & North (1948) that suspensions of organisms grown for 24 hr. on Bordet-Gengou medium gave good agglutination whilst after 72 hr. there was no agglutinating power at all. Suspensions of 24 hr. cultures kept overnight at 4° gave more marked agglutination than freshly prepared suspensions. Heating the suspension for 8 hr. at 65° caused a complete loss of the property to agglutinate. Treatment of the bacteria with formol (0.1-0.2 %) overnight caused loss of the agglutinating power. Similar treatment with phenol (0.1-0.5 %) and thiomersalate (1/5000-1/50,000) had no such effect.

We found the anticoagulants heparin, citrate and oxalate to be equally suitable in the test. Repeated washing of the red cells with excess saline produced no effect on the agglutination titres, but addition to the repeatedly washed cells of the original plasma was inhibitory to the test.

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#### **The Assay of Nisin.** By A. HIRSCH and M. J. R. HEALEY (*Reading*)

Nisin is an antibiotic which cannot be assayed by an agar diffusion technique. A dilution method may be used, or an assay can be based on the rapid bactericidal action of nisin, the number of surviving bacteria after a short period of contact being related to concentration. The best method to date is based on the observation that, in sublethal concentrations, nisin delays growth, the extent of the delay being dependent on the concentration. In a buffered medium, the pH is related to the time at which the growth of an acid-producing test-organism has started, and it is found that a linear relation obtains between the pH and the logarithm of the concentration over a range of 5-100 units/ml. Concentrations of less than 5 units/ml. cause a true increase in lag, while higher concentrations result in the appearance of resistant strains which grow more rapidly than the normal test-organism.

The accuracy of the 'lag-phase assay' compares favourably with established assay techniques. Using a 6-point design, a computing technique has been devised to give rapid estimations of potency with a statement of fiducial limits and a check on the validity of the assay. Details of this technique will be submitted for publication in the *Journal of General Microbiology*.

#### **Some Recent Applications of Nisin.** By A. HIRSCH and A. T. R. MATTICK (*Reading*)

The properties of an antibiotic, nisin, were described by Mattick & Hirsch (1947) and field trials against bovine mastitis have recently been made (Taylor, Hirsch & Mattick, 1949). Single udder infusions with volumes of 1 ml. per quarter

were used and 11 of 13 staphylococcal and 35 of 37 streptococcal cases treated with 5,000,000 units per quarter, were cured bacteriologically and clinically.

*In vivo* its bactericidal action against the tubercle bacillus was tested in a small experiment, rabbits infected with the bovine strain being treated for 5 days only by the intravenous route. There was a striking difference between treated and control animals. Macroscopically the former were normal, but the controls were heavily infected. Histologically, however, there was some evidence of infection in the treated animals.

*In vitro* human tubercle bacilli resistant to streptomycin are susceptible to nisin and vice versa. There is no synergism between the two antibiotics but there is synergism between nisin and sulphathiozole.

The level of toxicity of the crystalline material depends on the route of administration. Intramuscularly it is approximately the same as streptomycin. On prolonged use no obvious systemic effects are noted. Blood concentrations are well maintained and after the usual peak is passed small concentrations may persist for 48 hr.

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#### **Bacteriolytic Properties of Actinomycetes and Staphylococci.** By M. WELSCH and J. SALMON (*Liège*)

Soil actinomycetes dissolve heat-killed bacteria; they also inhibit the growth and lyse living Gram-positive bacteria. Culture-filtrates from these organisms (actinomycetin) may exhibit the same properties. Actinomycetin may retain its bacteriolytic activity towards living bacteria even after extraction of its bactericidal lipoids. Actinomycetes and actinomycetin are most active upon bacterial suspensions in water or water-agar, but inactive upon bacterial suspensions in nutrient broth or agar.

Staphylococci dissolve some heat-killed Gram-positive cocci added into nutrient-agar. Lysis is not obtained in water-agar and is reduced in diluted nutrient-agar. Bacteriolysis by, and autolysis of, staphylococci are enhanced under partial or complete anaerobic conditions. Autolysates from broth-cultures (autolysin) dissolve several heat-killed Gram-positive cocci, and also living staphylococci, in water but not in nutrient medium. Autolysin is inactive towards heat-killed Gram-negative bacteria.

During bacteriolysis by actinomycetes, actinomycetin, staphylococci or autolysin, it was not possible to demonstrate a *specific* loss of Gram-positiveness, apart from a general diminution of affinity for all basic dyes.

Oxidizing conditions and negativation of Gram's reaction do not appear as indispensable factors to permit a true lysis of Gram-positive bacteria.

**The Production of Ammonia by *Corynebacterium renale*. By R. LOVELL and D. G. HARVEY (Camden Town)**

*C. renale* causes a specific cystitis and pyelonephritis in cattle and lesions in experimental and natural cases of disease are localized in the medulla. The metabolism of the organism and especially its urease activity may have an important bearing on this selective localization.

*C. renale* produces ammonia from urea but not from any other common urinary nitrogenous substance; and of the many amino-acids tested only arginine produced significant quantities of ammonia. The arginase activity of *C. renale* was confirmed by isolation of ornithuric acid in a similar manner to that adopted by Hills (1940) working with *Streptococcus*, *Staphylococcus* and other pathogens; its identity as either a 'monohydrolase' or a 'dihydrolase' has not yet been confirmed. Serial investigations in ammonia production and growth of *C. renale* have shown that in the case of bovine urine and peptone water containing urea there was a steady decrease in growth and urea concentration with a parallel increase in ammonia. Peptone water, on the other hand, gave very little ammonia but the growth increased steadily.

## REFERENCE

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**Complexes Formed by Antibodies with Excess of Antigen and the Valency of Antibodies. By J. R. MARRACK, H. HOCH and R. G. S. JOHNS (White-chapel)**

From ultracentrifuge studies Pappenheimer, Lundgren & Williams (1940) concluded that the maximum valency of horse antitoxin for diphtheria toxin is greater than one and at most two. Heidelberger & Pedersen (1937) found that when the precipitate formed by egg albumin and rabbit antiserum was dissolved in a great excess of antigen most of the antibody-antigen complex sedimented with  $S_{20}=9$ ; from their data it seems that the molecular ratio of antigen to antibody was approximately one.

We have dissolved the precipitate, formed by horse-serum albumin and rabbit antibody, in excess of antigen. On electrophoresis in phosphate buffer at pH 8, ionic strength 0.1, two components were found with mobilities between those of albumin and  $\gamma$ -globulin. The amount of free albumin was calculated from the electrophoretic pattern. The amount of antibody in solution was known. The calculated ratio of bound antigen molecules to antibody molecules (assuming a molecular weight of 150,000) was more than one. We conclude that the faster complex contains two molecules of antigen to one of antibody and that this antibody is divalent.

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**Tetrathionase: Some Factors Influencing the Rate of Adaptive Enzyme Formation by Washed Bacterial Suspensions.** By W. H. H. JEBB, R. KNOX and A. H. TOMLINSON (*Oxford*)

The graph of enzyme content against time for a coliform organism adapting in washed suspension to tetrathionate reduction is the sigmoid curve typical of most microbial adaptations. Adaptation is affected by the following four factors:

(1) *Temperature.* 32° is the optimum. At higher temperatures adaptation is slower and less enzyme is produced. At 42° no adaptation occurs.

(2) *Concentration of substrate.* Adaptation appears to be slower at 0.0005 M than at 0.004 M, whereas the rate of reduction is constant down to 0.000125 M suggesting that in adaptation the substrate combines with some precursor rather than the enzyme.

(3) *Tryptic heart broth* added to adapting cells greatly increases the rate of adaptation and doubles the total amount of enzyme formed.

(4) *Pretreatment of cells* with mannitol and phosphate before adding the tetrathionate accelerates adaptation, without increasing the total enzyme formed. Although dinitrophenol inhibits the adaptation of untreated cells, pre-treated cells adapt slowly in its presence.

**The Part Played by an Adaptive Enzyme System in Bacterial Growth at Different Temperatures.** By R. KNOX (*Oxford*)

Cells of a tetrathionate-reducing organism grown in semi-anaerobic conditions can utilize tetrathionate as an alternative hydrogen acceptor to oxygen. Temperature is critical—in growth at 41° the cells gain no advantage from tetrathionate, whereas at 34–37° they grow much better with than without it. This difference is not due to serious interference with growth at the higher temperature, since in well-aerated broth the growth rate is practically unaffected between 34 and 41° both with and without tetrathionate. For the formation of nitratase in growth temperature seems much less critical. The greater sensitivity to temperature of tetrathionase is unlikely to be due entirely to increased toxicity of the substrate at the higher temperature, since enhanced growth due to adaptive formation of nitratase still occurs even in the presence of tetrathionate at 42°—at which temperature tetrathionase formation is completely suppressed. It seems, therefore, that adaptive formation of tetrathionase during growth is much more sensitive to temperature than either nitratase formation or 'growth' itself.



**DEMONSTRATIONS**

J. UNGAR and A. M. JAMES (*Greenford*). 'Factors affecting the agglutmability of red cells by *Haemophilus pertussis*.'

A. VOUREKA and A. FLEMING (*Paddington*). 'Staining of flagella.'

W. H. HUGHES, I. R. H. KRAMER and A. FLEMING (*Paddington*). 'Morphological changes in some bacteria grown in presence of penicillin.'

G. BELYAVIN (*Hampstead*). 'Phase-contrast microscopy and Orskov's method of demonstrating flagellar movement.'





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